ANTIBIOFILM ACTIVITY FROM NOVEL SOIL BACTERIAL SPECIES OF *PAENIBACILLUS HAEMOLYTICUS* STRAIN 139SI TOWARDS NEW THERAPEUTIC MANAGEMENT OF CHRONIC AND RECURRENT TONSILLITIS

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Field of Study: Clinical Bacteriology

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ABSTRACT

A biofilm is a layer of microbial cells that is firmly attached to surfaces and enclosed in a matrix of polysaccharide material. Biofilms pose a problem to the environment, industry and the medical field causing a variety of chronic infections particularly in the ear, nose and throat like chronic and recurrent tonsillitis. Therefore, development of new therapeutic strategies against biofilm-forming pathogens is essential in the management of biofilm-associated tonsillar diseases. One hundred and forty (140) palatine tonsils were collected from 70 patients undergoing tonsillectomy at University Malaya Medical Centre (UMMC) and the type of microorganisms and their antimicrobial susceptibility of both swab and biopsy tonsillar specimens were identified. The presence of bacterial biofilms in the excised tonsils was detected via Scanning Electron Microscopy and Confocal Laser Scanning Microscopy, and the ability of tonsillar bacterial isolates to form biofilms was determined via Congo Red Agar method and Microtiter Plate assay. A culture filtrate from novel soil bacterial species of *Paenibacillus haemolyticus* strain 139SI was tested for its acute toxicity in rats and evaluated for its antibiofilm activity both *in vitro* via Microtiter Plate assay and *in vivo* using a rat model of chronic lung infection. The soil bacterial filtrate was purified via High Performance Liquid Chromatography, and the selected compounds were tested against clinical isolates for their antibiofilm activity. The most potential antibiofilm compounds were further analysed via Liquid Chromatography-Mass Spectrometry. Two main groups of clinical cases were classified as infected tonsils represented by 49 (70%) cases of recurrent tonsillitis, 9 (12.85%) cases of chronic tonsillitis in addition to non-infected (hypertrophied) tonsils represented by 12 (17.14%) cases of obstructive sleep apnea. The presence of bacterial biofilms was detected in 42 (60%) patients.

A total number of 464 bacterial isolates were recovered from tonsillar specimens with 184 (39.65%) *Staphylococcus aureus* isolates as the most common followed by 86 (18.53%) of *Haemophilus influenzae* isolates. Three susceptibility patterns were found among *S. aureus* isolates with 89.4% being susceptible to all tested antimicrobial agents, 10.6% resistant to fusidic acid and 0.5% resistant to both methicillin and fusidic acid. The antimicrobial agent co-trimoxazole showed the highest rate of resistance among all bacterial isolates including 55 (98.2%) isolates of Group B Streptococci and 11 (78.5%) isolates of Group A Beta-Haemolytic Streptococci, 14 (56%) isolates of Group G Streptococci, 10 (32.2%) isolates of *Haemophilus pararinfluenzae* and 27 (31.3%) isolates of *Haemophilus influenzae*. The soil bacterial filtrate of *Paenibacillus haemolyticus* strain 139SI showed no signs of toxicity and a significant antibiofilm activity was detected in the lungs of chronically-infected rats. Four potential antibiofilm compounds of the bacterial filtrate namely FR4, FR5, FR8 and FR13 were identified. The compound FR5 with a molecular weight of 253.237 and a molecular formula of $C_8H_{20}N_3O_4P$ exhibited the strongest antibiofilm activity. In conclusion, discovery of an antibiofilm activity from a novel soil bacterial species of *Paenibacillus haemolyticus* strain 139SI adds an important dimension in the search for new potent compounds against biofilm infections.

ABSTRAK

Biofilm merupakan satu lapisan sel-sel mikrob yang melekat pada permukaan dan terbenam dalam bahan matriks polisakarida. Biofilm mewakili masalah kepada alam sekitar, industri dan bidang perubatan menyebabkan pelbagai jangkitan kronik terutama di telinga, hidung dan tekak seperti tonsilitis kronik dan berulang. Biofilms mewakili masalah alam sekitar, industri dan dalam bidang perubatan, ia menyebabkan pelbagai jangkitan kronik terutama di telinga, hidung dan tekak seperti tonsilitis kronik dan berulang. Oleh itu, pembangunan strategi baru terapeutik terhadap patogen pembentukan-biofilm adalah penting di dalam pengurusan bioflm-penyakit berkaitan tonsillar. Seratus empat puloh (140) tonsil palatin telah dikumpulkan daripada 70 pesakit yang menjalani pembedahan tonsilektomi di Pusat Perubatan Universiti Malaya (PPUM) dan telah mengenal pasti jenis mikroorganisma dan kerentanan antimikrobial mereka bagi kedua-dua swab dan spesimen biopsi tonsillar. Kehadiran biofilm bakteria dalam tonsil dibuang telah dikesan melalui Pengimbasan Mikroskop Elektron dan Laser Confocal Mikroskop Pengimbasan, dan keupayaan isolat bakteria tonsillar membentuk biofilm telah ditentukan melalui kaedah Agar Merah dan asai Microtiter Plat. Turasan kultur dari sepsis novel bacteria tanah *Paenibacillus haemolyticus* strain 139SI telah diuji untuk ketoksikan akut pada tikus dan telah dinilai untuk aktiviti antibiofilm secara *in vitro* melalui asai Mikrotiter Plat dan *in vivo* dengan menggunakan model tikus jangkitan paru-paru kronik. Turasan bakteria tanah telah ditulenkan melalui Kromatografi Cecair Prestasi Tinggi, dan sebatian yang dipilih telah diuji terhadap isolat klinikal untuk aktiviti antibiofilm mereka. Sebatian antibiofilm yang paling berpotensi seterusnya dianalisis melalui Kromatografi Cecair Spektrometri Jisim. Dua kumpulan utama kes-kes klinikal

telah diklasifikasikan sebagai tonsil yang dijangkiti adalah diwakili oleh 49 (70%) kes tonsilitis berulang dan 9 (12.85%) kes tonsilitis kronik, dan sebanyak 12 (17.14%) kes adalah tonsil yang tidak dijangkiti (hypertrophied) diwakili oleh apnea tidur obstruktif. Kehadiran biofilm bakteria telah dikesan dalam 42 (60%) orang pesakit. Sejumlah 464 isolat bakteria telah diasingkan dari spesimen tonsillar dan telah didapati *Staphylococcus aureus* mempunyai jumlah terbanyak iaitu 184 (39.65%) isolat dan diikuti dengan *Haemophilus influenzae* sebanyak 86 (18.53%) isolat. Tiga corak kerentanan telah ditemui di kalangan isolat *S. aureus* di mana sebanyak 89.4% adalah sensitive kepada semua agen antimikrobial yang diuji, 10.6% isolat *S. aureus* mempunyai kerintangan terhadap antibiotik asid fusidik dan 0.5% kerintangan terhadap antibiotik metisilin. Agen antimikrobial co-trimoxazol telah menunjukkan kadar kerintangan tertinggi di kalangan semua isolat bakteria dan ini termasuklah 55 (98.2%) isolat Streptococci Kumpulan B, 11 (78.5%) isolat Streptococci Beta-hemolitik Kumpulan A, 14 (56%) isolat Streptococci Kumpulan G, 10 (32.2%) isolat *Haemophilus pararinflunzae* dan 27 (31.3%) isolat *Haemophilus influenzae*. Turasan bakteria tanah *Paenibacillus haemolyticus* strain 139SI tidak menunjukkan tanda-tanda sebarang ketoksikan dan terdapat aktiviti antibiofilm yang ketara telah dikesan di dalam paru-paru tikus yang dijangkiti secara kronik. Empat sebatian antibiofilm berpotensi dari turasan bacteria telah dinamakan sebagai FR4, FR5, FR8 dan FR13. Sebatian FR5 mempunyai activiti antibiofilm paling tinggi dan berat molekulnya adalah 253.237 dengan formula $C_8H_{20}N_3O_4P$. Kesimpulannya, dengan penemuan aktiviti antibiofilm dari spesis novel bakteria tanah *Paenibacillus haemolyticus* strain 139SI akan menambah satu dimensi penting untuk carian sebatian baru berpotensi terhadap jangkitan biofilm.

DEDICATION

To my father, whose wisdom continues to inspire me,

To my mother, whose love continues to nourish me,

To my siblings, who are always there for me

Thank you

I love you all

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Indeed, the academic and technical staff of Clinical Diagnostic Laboratory (CDL) at University Malaya Medical Centre (UMMC) deserves an acknowledgement for their input in isolating and identifying our clinical bacterial isolates. Moreover, the Tropical Infectious Diseases Research & Education Centre (TIDREC) at the Faculty of Medicine, University of Malaya deserves an appreciation for identifying our novel soil bacterial species of *Paenibacillus* according to the highest possible standards.

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LIST OF ABBREVIATIONS

ACUC	Animal Care and Use Committee
AHS	Alpha-Haemolytic Streptococci
AI	Adherence Index
AM	Ampicillin
AMC	Amoxicillin-Clavulanic acid
AN	Amikacin
ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
AZM	Azithromycin
BB	Bright Black
BHI	Brain Heart Infusion
BIC	Biofilm Inhibitory Concentration
BLNAR	Beta-Lactamase Negative Ampicillin-Resistant
BLPB	Beta-Lactamase-Producing Bacteria
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
CDL	Clinical Diagnostic Laboratory
CF	Cystic Fibrosis
CFP	Cefoperazone
CFS	Cell-Free Supernatant
CFU	Colony Forming Units
CHOC	Chocolate Agar
CIP	Ciprofloxacin
CLSI	Clinical Laboratory and Standards Institute
CLSM	Confocal Laser Scanning Microscopy
CM	Clindamycin
ConA	Concanavalin A
CRA	Congo Red Agar
CT	Chronic Tonsillitis
CTR	Ceftriaxone
CTX	Cefotaxime
CV	Crystal Violate
CXM	Cefuroxime
DPX	Dibutyl Phthalate Xylene
EM	Erythromycin
ENT	Ear, Nose and Throat
EPS	Exopolysaccharide
FA	Fusidic Acid
FAA	Fastidious Anaerobic Agar

FESS	Functional Endoscopic Sinus Surgery
GABHS	Group A Beta-Haemolytic Streptococci
GBS	Group B Streptococci
GCS	Group C Streptococci
GFS	Group F Streptococci
GGS	Group G Streptococci
GM	Gentamicin
GNB	Gram-Negative Bacilli
H&E	Haematoxylin and Eosin
HI	*Haemophilus influenzae*
HPLC	High Performance Liquid Chromatography
HTM	Haemophilus Test Medium
I	Intermediate
KIA	Kligler Iron Agar
KP	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* Carbapenemase
LC-MS	Liquid Chromatography-Mass Spectrometry
LEX	Cephalexin
LIMP	Lung Index of Macroscopic Pathology
LM	Light Microscopy
MALT	Mucosa-Associated Lymphoid Tissue
MDR	Multidrug Resistant
MDR-GNB	Multidrug Resistant Gram-Negative Bacilli
ME	Methicillin
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
MR	Methyl Red
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
MTP	Microtiter Plate
NPLH	Nasopharyngeal Lymphoid Hyperplasia
NST	Non-Group A Streptococcal Tonsillitis
OB	Opaque Black
OCT	Optimal Cutting Temperature
OME	Otitis Media with Effusion
OSA	Obstructive Sleep Apnea
PA	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEN	Penicillin
PI	Propidium Iodide
PIA	Polysaccharide Intercellular Adhesin
PPD	*para*-phenylenediamine
PS	Primary Snoring
PT	Palatine Tonsil

QOL	Quality Of Life
QS	Quorum Sensing
QSI	Quorum Sensing Inhibition
R	Resistant
RA	Rifampin
RT	Recurrent Tonsillitis
S	Susceptible
SA	*Staphylococcus aureus*
SAM	Ampicillin-Sulbactam
SD	Sprague Dawley
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SEM	Standard Error Mean
SP	*Streptococcus pyogenes*
SPn	*Streptococcus pneumoniae*
SPSS	Statistical Product and Service Solutions
ST	Group A Streptococcal Tonsillitis
SXT	Trimethoprim-Sulfamethoxazole
T&A	Tonsillectomy and Adenoidectomy
TEM	Transmission Electron Microscopy
TH	Tonsillar Hypertrophy
THIO	Thioglycollate Broth
TLRs	Toll-Like Receptors
TOF	Time Of Flight
TSB	Trypticase Soy Broth
TZP	Piperacillin-Tazobactam
UMMC	University Malaya Medical Centre
UPLC–DAD	Ultra-Performance Liquid Chromatography-Diode Array Detection
UPLC-ESI-MS	Ultra-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
URI	Upper Respiratory Tract Infection
VA	Vancomycin
VGS	Viridans Group Streptococci
VP	Voges-Proskauer test
WR	Waldeyer's Ring

CHAPTER ONE

INTRODUCTION

1.1 Definition of Biofilms

Majority of the living biomass on earth is determined by the biological activity of microorganisms (King *et al.*, 2009). Basically, any accumulation of living microbes on a surface is typically referred to as biofilm or slime (Bott, 2011). Biofilms can be defined as complex microbial communities consisting of either single bacterial or fungal species or even multiple diverse species that are attached to a surface (Dowd *et al.*, 2008). These microorganisms synthesize and secrete slimy or sticky substances that anchors the biofilm structure firmly to living (biotic) or non-living (abiotic) surfaces (Costerton *et al.*, 1995; Stoodley *et al.*, 2002). These biofilms are considered dynamic hydrated structures distributed widely throughout the natural, industrial and medical settings that can have dangerous and costly effects (Cooper and Okhiria, 2006).

Bacteria are considered the best example of microorganisms with respect to surface attachment and biofilm formation (Lindsay and von Holy, 2006). Traditionally, it was known that bacteria are growing in free-living or planktonic populations (Fux *et al.*, 2003). However, in natural ecosystems bacteria tend to form communities of sessile cells embedded in a self-produced extracellular matrix (ECM) forming what is called a biofilm (Costerton *et al.*, 1999). The most important characteristic of a biofilm structure is the production of ECM, which is a complex of proteins, nucleic acids and extracellular polymeric substances or exopolysaccharide (EPS) (Seminara *et al.*, 2012). The different definitions of bacterial biofilms differ mainly in whether the cells have to be attached to a surface or whether the bacteria exist in a structured community (Bjarnsholt, 2011). The classical definition of bacterial biofilms is aggregations of

bacterial cells that are attached to biotic or abiotic surfaces and enclosed in a self-produced matrix of extracellular polymeric substance (EPS) and other elements depending on the environment they are growing in (Donlan, 2002). In the medical field, a biofilm is defined as a layer of sessile cells embedded in an exopolysaccharide matrix that is resistant to most antimicrobials and host defences (Bjarnsholt, 2011).

Because biofilm-associated bacteria differ phenotypically from their free-living counterparts (Donlan, 2002), the new definition must take into consideration other physiological characteristics like the altered growth rate and the expression of specific genes that planktonic bacteria do not (Donlan and Costerton, 2002), which leads us to a unified definition of bacterial biofilms as the predominant life form of all bacterial species in their natural habitat whether environmental or pathogenic (Wolcott and Ehrlich, 2008). At the most basic level a biofilm can be described as bacteria embedded in a thick, slimy barrier of sugars and proteins that protects the bacteria from external threats (Phillips *et al.*, 2010). Our simplified definition of a biofilm is a slimy layer of microbes attached to a surface.

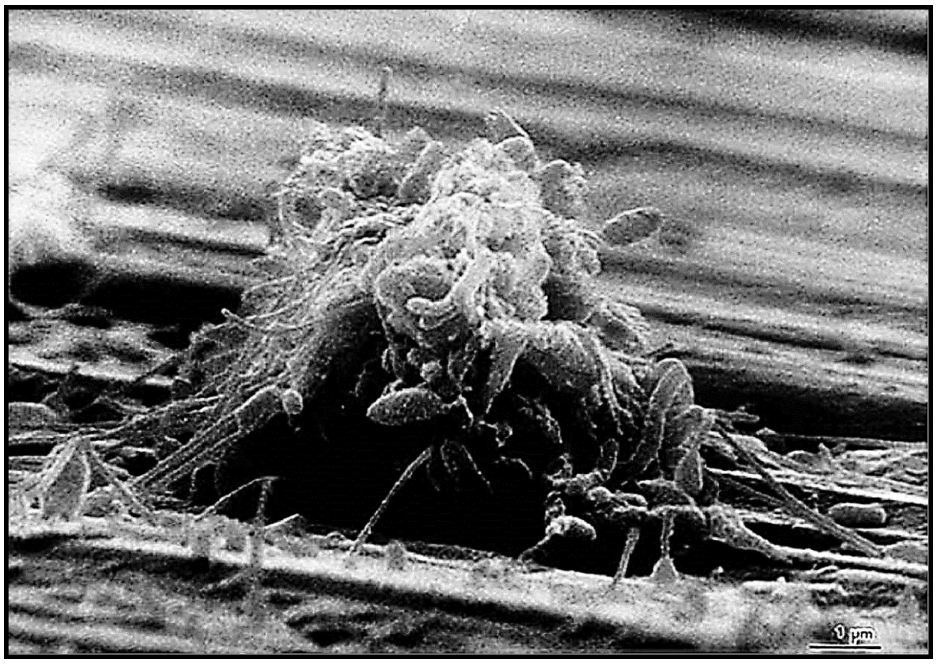

Figure 1.1 Scanning electron micrograph of a biofilm on metal surface. Adapted from (Donlan and Costerton, 2002).

1.2 Historical Background of Biofilms

Since the early days of microbiology up until the middle of the twentieth century, bacteria were viewed as being free-floating single cells referred to as the planktonic state (Atlas and Bartha, 1997). However, the study of bacterial biofilms goes back to the seventeenth century when Dutch microbiologist Antonie van Leewenhoek examined for the first time in 1684 the accumulation of microorganisms in a dental plaque on his own teeth calling it animalcules (Donlan and Costerton, 2002). He observed that the animalcules (bacteria) inside the previously mentioned plaque were protected against vinegar in contrast to the bacteria outside the plaque, which explains one of the major hallmarks of biofilms, i.e. tolerance against antimicrobial agents (Bjarnsholt, 2011). However it was only around the mid 1920's when Winogradsky, Cholodny and Conn made an important observation in biofilm research stating that bacteria growing on soil-submerged glass slides for a specific time interval are different from those they cultured from seawater (Lappin-Scott, 1999).

One of the pioneers of biofilms research is ecologist Claude ZoBell (Lappin-Scott, 1999) who in 1933 described the concept of marine bacterial attachment to submerged slide surfaces (ZoBell and Allen, 1933) and designed an apparatus to study bacterial attachment to surfaces (ZoBell and Allen, 1934). Other findings by ZoBell included the presence of bacteria as sessile phase rather than free-living planktonic phase on the surface of a sterile glass with seawater inside (ZoBell, 1936). In 1933, Henrici presented for the first time photomicrographs of bacterial aggregations describing the water bacteria as non-free floating organisms growing upon submerged surfaces (Henrici, 1933). Another contribution to biofilm research was achieved in 1940 when Heukelekian and Heller reported that surface characteristics are important factors for bacterial adhesion (Heukelekian and Heller, 1940) which represent a crucial factor in the control of infectious caused bacterial biofilms (Cortés *et al.*, 2011). The field of

environmental microbiology accepted the concept of bacterial aggregations almost 20 years before medical field (Bjarnsholt, 2011). Due to the fact that bacterial aggregations have long been used in waste water treatment plants, and the first article using the term biofilm was by Rogovska *et al.* that was published in 1961 in Mikrobiologiya (Bjarnsholt, 2011).

In 1971, Marshall provided evidence via Scanning Electron Microscopy (SEM) that attached bacteria were associated with the surface by fine extracellular polymeric fibrils (Marshall *et al.*, 1971). Moreover, in 1976, a correlation between the biological and physiological properties of bacteria to their behaviour in natural habitats was reported (Marshall, 1976). In 1977, electron microscopic observations showed that bacteria within a biofilm were embedded in a fibrous highly hydrated exopolysaccharide matrix whose chemical composition was species-specific (Sutherland, 1977). The medical field started to describe clumps of bacteria in 1977, then Høiby *et al.*, described the aggregations of *Pseudomonas aeruginosa* in patients with chronically infected lungs (Hoiby, 1977). However, the concept of bacterial biofilm was not practically established until 1978 when Costerton presented for the first time the unified theory of bacterial biofilm formation which stated that in all aquatic systems with sufficient concentrations of nutrients, sessile populations of bacteria form glycocalyx-embedded structures called biofilms that are adherent to surfaces in medical, natural, and industrial ecosystems (Costerton *et al.*, 1978).

In the late 1980s, advances in the field of light microscopy by the invention and use of Confocal Laser Scanning Microscopy (CLSM) led scientists to realize that bacterial biofilms consist of microcolony communities (Costerton *et al.*, 1999) and that attachment to a surface is a key element for colonization and biofilm formation (Whittaker *et al.*, 1996). New aspects of biofilms were investigated in 1990 such as the characteristics of spatial and temporal heterogeneity and involvement of inorganic or

abiotic substances in the biofilm matrix. (Characklis and Marshall, 1990; Donlan and Costerton, 2002). In 1991, the use of CLSM revolutionized the study of biofilms (Costerton *et al.*, 1994) by reporting that sessile bacteria grow in matrix-enclosed communities inter-connected with less-dense regions of permeable water channels (Caldwell *et al.*, 1992; Lawrence *et al.*, 1991). However, it was only in 1993 when the American Society for Microbiology (ASM) recognized the biofilm mode of growth as an important bacterial trait (Bjarnsholt, 2011).

In 1995, Costerton *et al.* stated that bacterial adhesion triggers the expression of certain genes that controls the production of specific components necessary for biofilm formation (Costerton *et al.*, 1995) and that the process of biofilm formation is regulated by specific genes transcribed during the initial stage of cell attachment (Costerton and Lappin-Scott, 1995; Donlan and Costerton, 2002). In light of all these findings, our knowledge and understanding of biofilms has expanded which will help us explore new strategies for a better management of bacterial biofilms in various fields

1.3 Formation of Biofilms

The formation of a three-dimensional biofilm structure involves a coordinated series of molecular events controlled by an inter-bacterial communication mechanism called Quorum Sensing (QS) (Davies *et al.*, 1998). It is understood now that bacteria undergo many changes during their transition from planktonic to sessile organisms forming the community of a biofilm (O'Toole *et al.*, 2000). However, the biofilm phenotype and the rate of microbial attachment to a surface or substratum depends on many factors including the type of organism and its cell surface structures such as fimbriae, EPS, and flagella (Donlan, 2002), the type of substratum (Costerton and Lappin-Scott, 1995), the rate of liquid flow over the surface (Brown and Smith, 2003)

and characteristics of the aqueous medium such as pH, nutrient levels (Cowan *et al.*, 1991), ionic strength (Fletcher, 1988) and temperature (Donlan *et al.*, 1994). The development and formation of bacterial biofilms occurs in different stages depending on the condition of the attachment surface (Lindsay and von Holy, 2006), it can be categorized into five stages represented by reversible or initial attachment, irreversible attachment, maturation-1 or colonization, maturation-2 and detachment or dispersion (Sauer *et al.*, 2002).

The first stage of biofilm formation is represented by the initial or reversible attachment of bacterial cells to a surface which occurs via different mechanisms including sedimentation (van Loosdrecht *et al.*, 1990), active movement and electro-kinetic or thermodynamic properties (Flemming *et al.*, 1998) such as electrostatic charge (Fletcher, 1987), hydrophobicity (Allison *et al.*, 1990) and surface free energy (Van der Mei *et al.*, 1992). This type of non-specific attachment is considered the weakest point in connecting the cells to surface (Busscher *et al.*, 1995) and it will lead eventually to a balance distribution between the adhering and non-adhering cells (van Loosdrecht *et al.*, 1990).

The second stage is the irreversible attachment were bacterial cells are being stimulated via membrane-bound sensory proteins leading to the production of exopolysaccharide (Boyd and Chakrabarty, 1995) that triggers the formation of cell-to-cell bridges that glue cells to the surface (Donlan, 2002; Hall-Stoodley *et al.*, 2004). The third stage in the establishment of biofilm is colonization or maturation-1 (Zottola and Sasahara, 1994) were bacterial cell density increases and the population reaches a critical threshold leading them to communicate via secretion of low molecular weight signals (Davies *et al.*, 1998), these signals eventually trigger the expression of virulence factors in a process called quorum sensing (Pearson *et al.*, 1995; Schauder and Bassler,

2001). Bacteria start to grow and form microcolonies that are considered the building blocks of the biofilm structure (Costerton *et al.*, 1994).

As the biofilm structure starts to grow, the fourth stage which is maturation-2 begins when cell clusters reach their maximum thickness (Davies *et al.*, 1998) exhibiting a complex architecture comprising of microcolonies embedded in a matrix of EPS and separated by permeable water channels that are used for the circulation of nutrients and transport of waste products (Costerton *et al.*, 1994; Stoodley *et al.*, 1994). The formed biofilm will eventually find its way to stabilize the interactions between the bacterial species through changes in their symbiotic relationships leading to alter the function of the whole biofilm population (Hansen *et al.*, 2007).

The fifth and final stage is the detachment or dispersion of bacterial cells from the biofilm structure, this stage is considered highly important due to its role in spreading new colonizers into neighbouring sites (Stoodley *et al.*, 2001) leading to contaminate and infect both industrial (Zottola and Sasahara, 1994) and clinical settings (Nickel *et al.*, 1994). Based on the frequency and area of detachment (Bryers, 1988), bacterial cells detach from their biofilms via erosion which is a detachment of single cells and sloughing which is a loss of biofilm mass (Characklis, 1990). The detachment of cells from the biofilm structure is a complicated process (Purevdorj *et al.*, 2002) because it must include a signal that triggers the release of certain enzymes that can degrade the components of biofilm's matrix as well as the conversion of cells into the planktonic phenotype (Costerton, 2007). An example of detachment enzymes is the lyase enzyme in *P. aeruginosa* which is synthesized and stored in the periplasmic space (Boyd and Chakrabarty, 1994) and is released to digest the matrix (alginate) upon detachment (Costerton, 2007). A simplified description of the biofilm life cycle can be summarized into three major steps as shown in (Figure 1.2).

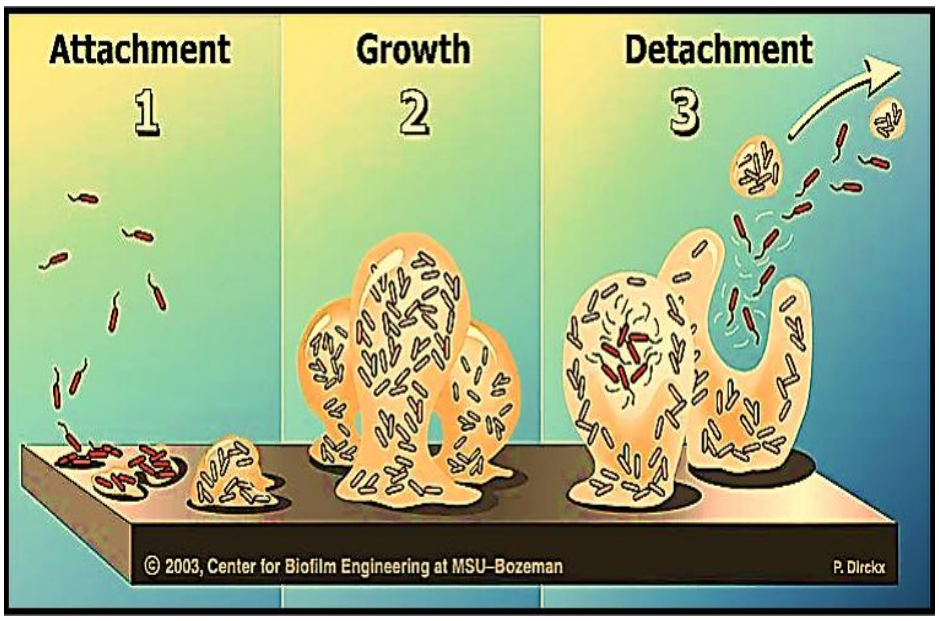

Figure 1.2 The three essential steps of biofilm formation. Adapted from (Stoodley and Dirckx, 2003).

1.4 Biofilms in Nature and Industrial Settings

Microbial biofilms are considered vital components of the natural environment that can cause both beneficial and harmful effects (Centre for Biofilm Engineering, 1990). Bacteria within biofilms have the ability to form on a wide range of surfaces including natural aquatic ecosystems, industrial water piping, living tissues and indwelling medical devices (Donlan, 2002). In natural environments, biofilms can negatively affect the environment via consuming atmospheric gases that can change the climate, mobilizing toxic elements and creating oxygen depletion zones in areas like lakes, rivers and coastal environments (Center for Biofilm Engineering, 1990). This is mainly due to the fact that biofilm bacteria are more adaptable to survive certain threats like bacteriophages and amoebas in comparison to their free-living counterparts (Costerton *et al.*, 1999). In industrial settings, biofilms or biofouling shows resistance to chemical biocides making them hard to remove (Costerton *et al.*, 1995).

The challenges associated with the accumulation of bacteria on the surfaces of industrial processing equipment is represented by blockage in water circuits that leads to increased pressure loss, reduced heat transfer in heat exchangers (Whitehouse *et al.*, 1986), interference with the function of chemical additives and surface corrosion of equipment through depolarisation of oxygen that encourages the growth of anaerobic bacteria (Bott, 2011). Because bacterial biofilms cause serious problems in industrial water systems, developing methods to sample and monitor those biofilms could make a major contribution in reducing climate change, equipment damage and product contamination (Donlan and Costerton, 2002).

The cost of unwanted biofilm accumulation on the surface of industrial equipment can represent a large proportion of the total operating cost (Bott, 2011). Many attempts have been made at the stage of equipment design to reduce such operating problems, for example the idea of making the equipment larger to overcome the fouling problem or the type of construction material used should be more expensive to reduce the microbial accumulation and potential corrosion problems. However, these attempts will eventually increase capital costs (Bott, 2011). The presence of biofilms will reduce the flow area and will create a rough surface to the fluid flow in equipment such as heat exchangers, therefore the pumping energy requirement and the associated cost will become higher in addition to the large carbon dioxide emission from combustion process that leads to significant climate changes that will add more problems to the global warming phenomenon (Bott, 2011). Even though the potential problems due to biofouling may have been recognised at the design stage, it may still be necessary to take effective approaches to prevent or restrict the development of biofilms on the surfaces of industrial equipment.

On the other hand, there are many potential applications of microbial biofilms in industry such as bioremediation (Singh *et al.*, 2006), production of fine chemicals (Li, *et*

al., 2006), fermentation (Kunduru and Pometto, 1996), biofiltration (Cohen, 2001), wastewater treatment (Nicolella *et al.*, 2000), biofuel production (Wang and Chen, 2009) and generation of electricity in microbial fuel cells (Rabaey *et al.*, 2007). An example of the beneficial effects of bacterial biofilms is the reduction of acid water drainage by establishing a biofilm biobarrier to keep oxygen from reaching subsurface water (Costerton and Dirckx, 1996).

1.5 Biofilms in Health and Medicine

From a medical perspective, both commensal and pathogenic microorganisms form biofilm-like structures that are associated with epithelial or endothelial lining, attachment on teeth or medical implant surfaces, intestinal or vaginal mucus layer or even being embedded in the lung tissue (Bryers, 2008). Biofilm formation has a negative impact on health due to that fact that microorganisms growing within biofilms are significantly less susceptible to antibiotics and host defences than their planktonic counterparts (Bryers, 2008). Modern medicine is facing the spread of bacterial biofilm infections (Fux *et al.*, 2005). Many biofilm infections are notoriously difficult to resolve and are associated with chronic or recurrent infections. It has been reported that biofilm infections affect almost all tissues and structures in the human body including the ear, nose, throat, mouth, eye, lung, heart, kidney, gall bladder, pancreas, nervous system, skin, bone, as well as any implanted medical devices (Wolcott and Ehrlich, 2008). These infections represent many clinical challenges, including diseases with uncultivable species, chronic inflammation and rapidly acquired antibiotic resistance (Bryers, 2008). In addition to the fact that bacteria in a biofilm mode of growth are difficult to be detected in routine diagnostic labs in addition of being resistant to host immune defences (Fux *et al.*, 2005).

The Centres for Disease Control and Prevention (CDC) estimates that about 65% of all bacterial infections are associated with biofilms (Goodman *et al.*, 2011; Lewis, 2007), other literature report higher prevalence that can reach up to 80% (Hall-Stoodley *et al.*, 2004; Parsek and Singh, 2003). Thus, the impact of biofilm infections on healthcare is significant and requires immediate attention (Wolcott and Ehrlich, 2008). The attachment of bacteria on medical devices that are in contact with the human body provides a starting point for the onset of clinical infections (Renner and Weibel, 2011). Biofilm formation on various medically–related surfaces like surgical implants, catheters, and contact lenses affect their function and provides the opportunity for pathogenic bacteria to enter the human body and thus cause biofilm-associated infections (Bryers, 2008) through the production and release of endotoxins that will eventually trigger an immune response in patients (Donlan and Costerton, 2002).

1.6 Biofilm Infections

Advances in medical practice over the decades led to the proper management of acute bacterial infections (Costerton *et al.*, 2003), however another type of infectious diseases, i.e. chronic infections, is being recognized as more persistent over longer periods and highly resistant to common therapeutics and the host defences (Costerton *et al.*, 2003). It has been noticed that bacterial biofilms are the cause of various chronic infections including cystic fibrosis (CF), recurrent tonsillitis, chronic rhinosinusitis (CRS), chronic wound infections, chronic otitis media (OM), chronic urinary tract infections (UTI), periodontitis and device-associated infections (Hall-Stoodley and Stoodley, 2009). These infections are usually associated with pathogenic or opportunistic bacteria either as single species or as a polymicrobial community (Hall-Stoodley and Stoodley, 2009).

There are several common features for any chronic infection associated with biofilms, including a non-aggressive initial stage where an antibiotic therapy is usually prescribed (Wolcott and Ehrlich, 2008). These therapies typically treat the symptoms caused by planktonic cells detached from the biofilm yet it fails to kill the sessile cells within the biofilm itself (Marrie *et al.*, 1982). The next stage will be a severe subsequent infection that is again treated with antibiotics (Wolcott and Ehrlich, 2008). However, the infection will eventually get worse especially when treatment is stopped or withdrawn which might lead to the decision of removing the infected tissue or organ by surgery (Wolcott and Ehrlich, 2008). Biofilm infections are difficult to be cleared by the host defences even in individuals with a strong immune response (Khoury *et al.*, 1992). Therefore, the modern view to chronic infections requires multidisciplinary efforts to develop therapeutic agents that target sessile bacterial cells and their biofilm phenotype (Costerton *et al.*, 1999).

Common sites of primary biofilm infections are the mouth, catheter entries and implanted medical devices (Stoodley *et al.*, 2003). Once bacteria enter the circulatory system, they can reach all parts of the body causing secondary biofilm infections in sites such as the brain, kidneys, intervertebral spaces and the bones around implanted medical devices (Stoodley *et al.*, 2003). Examples of the possible entry points of biofilm bacteria are shown in (Figure 1.3).

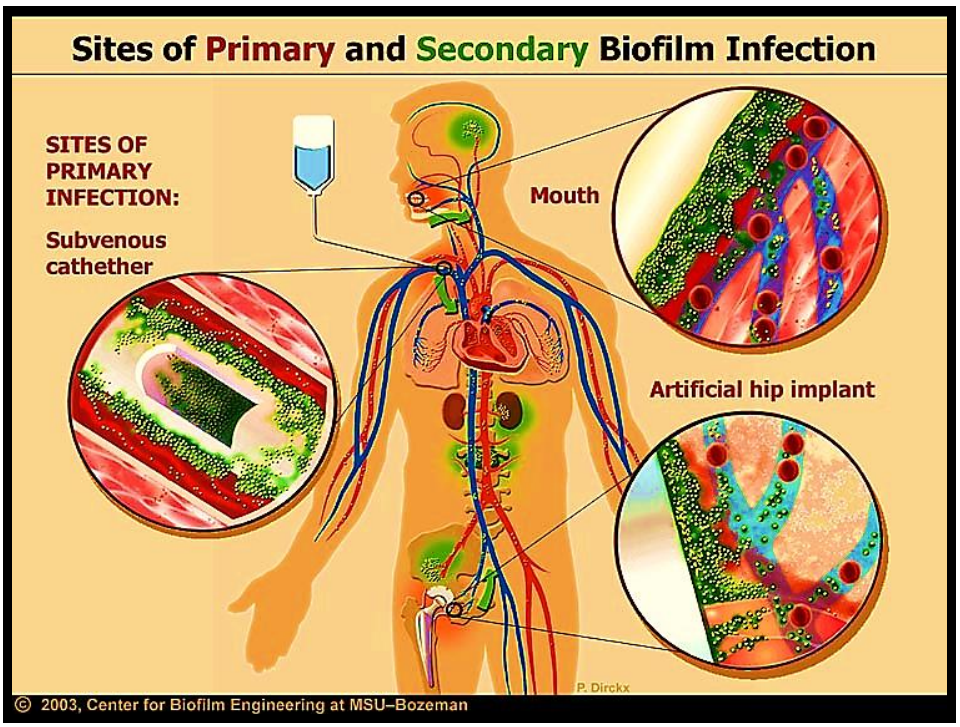

Figure 1.3 Sites of primary and secondary biofilm infections. Adapted from (Stoodley *et al.*, 2003).

1.6.1 Oral Infections

Bacterial biofilms of the oral cavity present a unique opportunity to investigate the inter-bacterial interactions and the impact of those interactions on host pathology (Palmer Jr. *et al.*, 2011). The oral cavity contains about 700 bacterial species, many of which form biofilms through sequential accumulations on tooth surfaces (Palmer Jr. *et al.*, 2011). Many of these are microbes are harmless, however some are pathogenic and can cause various infections (Costerton and Lange, 1999). In the absence of an adequate oral hygiene, ecological shifts occur within the microbial community which initiate two oral infections: caries and periodontal diseases (Palmer Jr. *et al.*, 2011). Dental caries or tooth decay result from a controlled metabolic process in the biofilm plaque that disturb the mineral balance at the biofilm-tooth interface leading to many clinical manifestations from white spot lesions to overt cavities and tooth destruction (Baelum

et al., 2008). Periodontal diseases are complex polymicrobial biofilm infections that involve interactions between oral bacteria and the host leading to accumulation of plaque at the gingival margin that eventually cause gingivitis (Palmer Jr. *et al.*, 2011). It has become clear that oral biofilm infections are considered the origin of many pathogenic organisms to disseminate into distant body sites causing various systemic diseases (Li *et al.*, 2000). An example of oral biofilm infections is illustrated in (Figure 1.4).

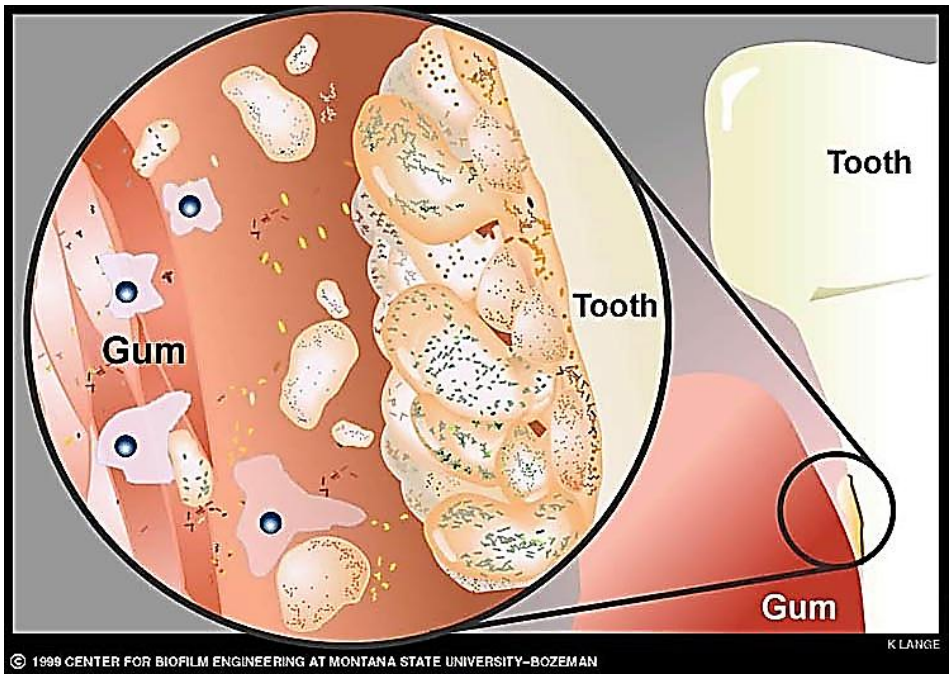

Figure 1.4 Oral biofilm infection forming a dental plaque Adapted from (Costerton and Lange, 1999).

1.6.2 Device-Associated Infections

Medical devices have gained a lot of importance in the past decades due to the higher demands of elderly patients suffering from degenerative diseases, the technological advances that can replace many functions of the body by such devices and the broad availability of health insurance in some countries (Darouiche, 2004; Zimmerli and Trampuz, 2011). However, implantation of medical devices is considered an

important source of nosocomial infections especially during the perioperative period (Roumbelaki *et al.*, 2008) due to the fact that bacteria can attach to non-living surfaces, including those of indwelling devices, and form biofilms that are highly resistant to antimicrobial treatment (Donlan, 2001). The pathogenesis of implant-associated infection involves interactions between the microorganism with the device that result in the formation of a biofilm and interactions of host defences with the implant that result in immunological response (Darouiche, 2001).

Biofilms on indwelling medical devices are composed of different bacterial species that may originate from the skin of patients or healthcare workers when entry ports are exposed (Donlan, 2001). One of the most common otolaryngological device-associated infections is ventilator-associated pneumonia which is correlated associated with injures on the tracheal epithelial surface during tracheal intubation (Pneumatikos *et al.*, 2009). These injures provide a rapid and direct access of bacteria from the upper into the lower respiratory tract allowing the biofilm formation on the surface of endotracheal tube (ETT) (Pneumatikos *et al.*, 2009). For a better understanding of biofilms on indwelling medical devices, development of reliable sampling and measurement techniques is crucial to investigate the role of biofilms in the pathogenesis of such infections (Donlan, 2001).

1.6.3 Wound Infections

Chronic wound infections represent a burden to healthcare systems causing significant morbidity and mortality to mankind (Kirketerp-Møller *et al.*, 2011). Biofilms in healed surgical wounds were first observed in 1985 when sutures were examined by scanning electron microscopy showing cocci bacteria encased in extracellular material and attached to the intradermal surface of the closures (Gristina *et al.*, 1985). Increasing

evidence has focused on the potential role of bacterial biofilms in the pathology of chronic wounds suggesting that biofilms are one of the aetiological factors in wound chronicity (Kirketerp-Møller *et al.*, 2011). This may explain why chronic wounds are difficult to heal despite adequate treatment and opens new paths for the search for more effective therapeutic approaches (Bjarnsholt *et al.*, 2008). Bacterial biofilms in the wound interfere with the host immune system leading to facilitate a chronic inflammation of the wound that will eventually prevent the healing process (Kirketerp-Møller *et al.*, 2011).

Treatment of biofilm wound infections has only temporary effect on both inflammation and healing due to the fact that bacteria in biofilm are up to 1000 times less susceptible to antibiotics and that the minimal inhibitory concentration (MIC) is not reached in the chronic wound fluid (Bjarnsholt *et al.*, 2007). Evidence obtained by molecular analyses of chronic wound specimens from patients with diabetic foot ulcers, pressure ulcers, and venous leg ulcers suggested diverse polymicrobial biofilms (James *et al.*, 2008). The prevalence of bacterial biofilms in specimens from chronic wounds relative to acute wounds provides an insight that biofilms may be more abundant in chronic wounds (James *et al.*, 2008). Therefore, understanding bacterial biofilms will provide more insights to discover and design new treatments for chronic wound patients (Kirketerp-Møller *et al.*, 2011).

1.6.4 Upper Respiratory Tract Infections

The upper respiratory tract (URI) including the ear, nose and throat (ENT) represent a natural habitat for a broad range of microorganisms such as commensal bacteria as well as potential pathogens (Garcia-Rodriguez and Fresnadillo Martinez, 2002). However, these bacteria can sometimes find ways to overcome the defence barriers of such locations and establish chronic infections that poses a challenge to both

medical practice and healthcare system (Morris, 2007). Infections in the URI such as acute otitis media, acute rhinosinusitis and acute pahryngotonsillitis are diseases that occur with extremely high frequency (Yamanaka, 2011). It has become clear that ENT chronic infections are becoming more resistant to common antimicrobial therapies (Vlastarakos *et al.*, 2007) due to the fact that bacteria may persist on mucosal surfaces through the formation of biofilms (Kania *et al.*, 2007).

Although most infections in otorhinolaryngology are of viral origins and are considered self-limited, bacterial infections are responsible for a significant degree of morbidity and have the potential to become life-threatening (Morris, 2007). The presence of biofilms in URT has been demonstrated in chronic otitis media, cholesteatoma, and chronic adenoiditis among other important diseases (Post *et al.* 2007). These biofilms play important roles resulting in their difficult eradication by means of resistance to immune clearance (Costerton *et al.*, 1987) and spreading new invaders to the surrounding tissues causing infections to recur and become chronic (Vlastarakos *et al.*, 2007). Moreover, the chronic nature of some ENT infections like chronic tonsillitis (CT), chronic rhinosinusitis (CRS), chronic otitis media (COM) and otitis media with effusion (OME) makes their diagnosis and management even more difficult (Vlastarakos *et al.*, 2007). The role of bacterial biofilm infections in the upper respiratory tract has been the subject of various papers due to their persistence, difficulties in culturing samples and resistance to therapeutic management (Morris, 2007). Such infections when occur in localized areas like the throat may either affect the mucosa of whole nasopharynx and oropharynx causing pharyngitis or may localize to the lymphoid tissue causing tonsillitis (Ludman, 1981).

1.6.4.1 Chronic and Recurrent Tonsillitis

The palatine tonsils (PT) are dense compact bodies of lymphoid tissue that are located in the lateral wall of the oropharynx (Kenna and Amin, 2009). It belongs to nasal-associated lymphoid tissue (NALT) that is part of a lymphatic tissue arrangement called Waldeyer's ring (WR) (Hellings *et al.*, 2000). Palatine tonsils are positioned at the openings of both digestive and respiratory tracts making them the first line of defence against inhaled and ingested microorganisms as shown in (Figure 1.5) (Perry and Whyte, 1998). The condition of tonsillitis, i.e. inflammation of the palatine tonsils, is considered one of the most common infectious diseases in children despite the widespread use of antibiotics (Chole and Faddis, 2003). It occurs when the activity of pathogens surpasses the activity of tonsillar lymphoid cells (Scadding, 1990).

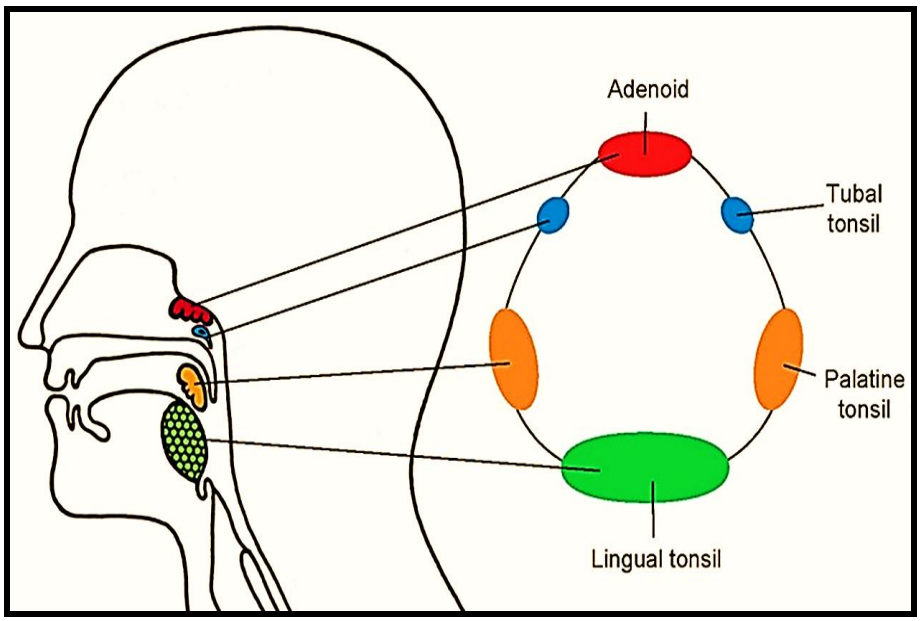

Figure 1.5 The lymphatic tonsillar tissue of Waldeyer's ring including the nasopharyngeal tonsil (Adenoid), tubal tonsils, palatine tonsils and lingual tonsil. Adapted from (Perry and Whyte, 1998).

Traditionally, Group A Beta-Haemolytic Streptococci (GABHS) has been considered the only important bacteria in the development of acute tonsillitis (Klein, 1975). However, there is increasing evidence that other bacteria can cause acute,

recurrent and even chronic tonsillitis such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae* (Brodsky *et al.*, 1988; Brook, 1987). Although these bacteria are classified as colonizers of the tonsils, they are now believed to become pathogenic in the presence of a recurrent bacterial infection (Brodsky, 1989). A clinical case of recurrent tonsillitis is shown in (Figure 1.6).

Chronic tonsillitis represents the most frequent lesion of the pharynx inflammatory pathology with multiple complications both locally and on distant sites (Gaffney and Cafferkey, 1998; Mattila *et al.*, 2001; Mogoanta *et al.*, 2008). It can be also the location of some specific infections such as tuberculosis and syphilitic lesions (Bourbeau, 2003). Chronic inflammation of the tonsils is usually caused by frequent attacks of acute tonsillar infections that fail to respond to therapy leading to cause scarring in the crypts of tonsils (Ludman, 1981). The fact that tonsillar infections may recur even when they are caused by organisms shown to be susceptible *in vitro* (Brook, 2001) led to the hypothesis that biofilm-forming bacteria within the crypts of chronically infected tonsils can resist both antibiotics and host defences (Chole and Faddis, 2003) and they may play a key role in the morbidity associated with tonsillar and/or adenotonsillar diseases (Al-Mazrou and Al-Khattaf, 2008).

Previous reports demonstrated that hypertrophied or enlarged tonsils are not necessarily an indication for tonsillectomy, i.e. surgical removal of tonsils, (Mattila and Tarkkanen, 1998) due to the fact that tonsils usually get enlarged during childhood and will eventually return back to their normal size in adolescence (Bergler *et al.*, 1999). Despite the high prevalence of chronic and recurrent tonsillitis among both paediatric and adults patients, a general agreement is still lacking in identifying the main causative organisms (Cowan and Hibbert, 1997) in addition to the insufficient data addressing the presence of biofilms on the surfaces of tonsils (Al-Mazrou and Al-Khattaf, 2008).

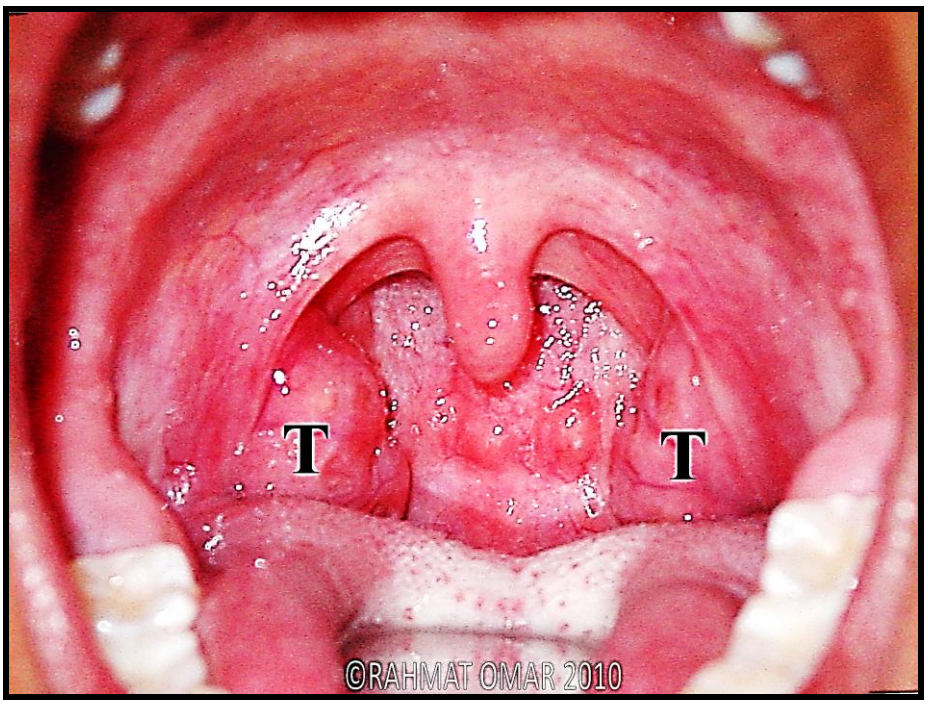

Figure 1.6 Clinical examination of a patient with recurrent tonsillitis. Curtsey of Dr. Rahmat Omar, University Malaya Medical Centre. T = enlarged palatine tonsil.

1.6.4.2 Chronic Adenotonsillits

The nasopharyngeal tonsil (adenoid) is a median mass lymphoid tissue that is located in the roof and posterior wall of the nasopharynx (Susan *et al.*, 2005). Both PT and adenoid are part of Waldeyer's ring providing defences through the production of immunoglobulins and the development of both B and T cells (Kenna and Amin, 2009). Adenotonsillar diseases, i.e. adenoiditis and recurrent tonsillitis, are common otolaryngological disorders caused by persistent bacterial infections (Zautner, 2012). Chronic adenotonsillar infection and/or hypertrophy are thought to be caused by resistant bacteria such as *Staphylococcus aureus*, *Haemophilus* sp. and *Streptococcus* sp. that persist within biofilm communities (Don *et al.*, 2005). The recurrent or chronic inflammation of the adenoids and tonsils leads to a chronic activation of both cellular and humoral immune response, resulting in enlargement or hypertrophy of the lymphoid tissue (Zautner, 2012). This hypertrophic tissue is the cause for various clinical

symptoms including obstruction of the upper airways, snoring and sleep apnea for adenoiditis or sore throat, dysphagia and halitosis for recurrent tonsillitis (Zautner, 2012). Tonsillar and adenotonsillar hypertrophy that cause obstructive sleep apnea (OSA) and/or chronic adenotonsillitis are still the commonest indications for adenotonsillectomy (T&A) (Al-Mazrou and Al-Khattaf, 2008). However, treatment strategies should target not only the removal of a mechanical obstacle of the airways but the elimination of a possible aetiological cause of a persisting biofilm infection (Zautner, 2012).

1.6.4.3 Chronic Otitis Media

The role of biofilms in the persistence of chronic, mucosal-based ENT infections was first recognized in otitis media (Post *et al.*, 2007). Chronic otitis media (OM) is one of the most common paediatric infections in which children receive antibiotics or undergo surgery (Hall-Stoodley *et al.*, 2006). Chronic OM includes both otitis media with effusion (OME) and recurrent otitis media (Hall-Stoodley *et al.*, 2006). Otitis media with effusion can result in conductive hearing loss, which has been linked to the delayed development of speech and socialization skills (Bennett *et al.*, 2001).

Chronic suppurative otitis media (CSOM) is one of the most common infections of childhood and is associated with significant morbidity (Roland, 2002; Verhoeff *et al.*, 2006). CSOM is usually caused by the presence of bacteria in the middle ear and mastoid cavity, which lead to the onset of a biofilm-associated chronic infection (Roland, 2002). These bacteria are introduced into the middle ear either from the external auditory canal through the perforation or from the nasopharynx though Eustachian tube (Dohar *et al.*, 2005). Microbiological studies of OME using highly sensitive techniques such as polymerase chain reaction (PCR) have demonstrated that traditional culturing methods are insufficient to detect the bacteria in OME (Post, 2001).

There is evidence that OME is associated with resistance to antibiotics which led to the hypothesis that biofilm-associated bacteria are involved in such infections (Ehrlich *et al.*, 2002). Moreover, the presence of biofilm bacteria on the surface of middle-ear mucosa rather than free-floating ones may explain the recalcitrant nature of OME (Post, 2001).

1.6.4.4 Chronic Rhinosinusitis

Chronic Rhinosinusitis (CRS) is one of the most common medical conditions affecting up to 16% of the population with an estimated cost of over $6 billion annually in the United States (Suh *et al.*, 2010). Patients with CRS demonstrate worse quality of life (QOL) scores than those suffering from other diseases like chronic obstructive pulmonary disease, congestive heart failure, back pain, or angina (Suh *et al.*, 2010). Despite the large socioeconomical impact of CRS, its pathophysiology remains largely unknown. One proposed aetiological factor of the chronic nature of this disease is the involvement of bacterial biofilms (Kilty and Desrosiers, 2008). The genetic shift in the biofilm bacteria as well as the multiple expressed phenotypes creates a complex community of pathogens, some of which will survive therapeutic treatments (Hunsaker and Leid, 2008). The challenges associated with the management of CRS patients may focus more on the biofilm present in the sinuses. However, it is important to mention that sinus cultures cannot be expected to provide a complete picture of the aetiology of chronic sinusitis. Therefore new diagnostic method and innovative treatment plans will be necessary to provide a lasting treatment of CRS (Hunsaker and Leid, 2008).

1.6.5 Lower Respiratory Tract Infections

Lower Respiratory Tract Infections (LRTI) are one of the leading causes of the morbidity and mortality in the world (Murray and Lopez, 1997). They are not a single

disease but a group of specific infections each with a different epidemiology, pathogenesis, clinical presentation and outcome (Mishra *et al.*, 2012). It has been suggested that there is a correlation between biofilm formation and signs and symptoms of lower respiratory tract infections (Chen *et al.*, 2007). Evidence explored the relationship between endotracheal tubes biofilms and lower respiratory tract infection via microscopic observation of biofilms on the surface of endotracheal tubes (ETTs) removed from neonates treated with intubated mechanical ventilation (Chen *et al.*, 2007). Examples of LRTIs that are biofilm-related; ventilator-associated pneumonia (VAP) and cystic fibrosis.

1.6.5.1 Cystic Fibrosis

Cystic Fibrosis (CF) represent the most common life-threatening autosomal recessive genetic disease among Caucasian children (Brussow, 2012). The life expectancy of most CF patients is determined by the development of a lung disease due to the failure in clearing thick mucus secretions from the lung that leads to chronic coughing followed by a frequent pneumonia infection (Brussow, 2012). The opportunistic pathogen *Pseudomonas aeruginosa* is significantly associated with the environment of CF-affected lungs where it can produce exopolysaccharide glycocalyx called alginate that makes the bacterium firmly adhere to lung epithelium that leads to the formation of a biofilm (Potera, 1999; Yan *et al.*, 2008).

Alginate is believed to increase resistance to both the host immune system and antibiotic therapy (May *et al.*, 1991). Evidence indicates that *P. aeruginosa* is more resistant to antibiotics when they are in the biofilm mode of growth, as they appear to be in the lungs of CF patients (May *et al.*, 1991; Wu *et al.*, 2004). It has been reported that 80%-90% of CF patients have biofilm of *Pseudomonas aeruginosa* in their lungs (Yan *et al.*, 2008). Microscopic investigations addressing the location of *P. aeruginosa* in CF

lungs revealed that destruction of CF lungs is correlated with the presence of mucus (alginate) produced by *P. aeruginosa* growing in biofilm layers on the respiratory zone, whereas non-mucoid bacteria are rarely observed (Figure 1.7) (Bjarnsholt *et al.*, 2009). This strongly suggests that the conductive respiratory zone serves as a reservoir for bacteria to grow as biofilms that is protected against antibiotics and host defences (Bjarnsholt *et al.*, 2009).

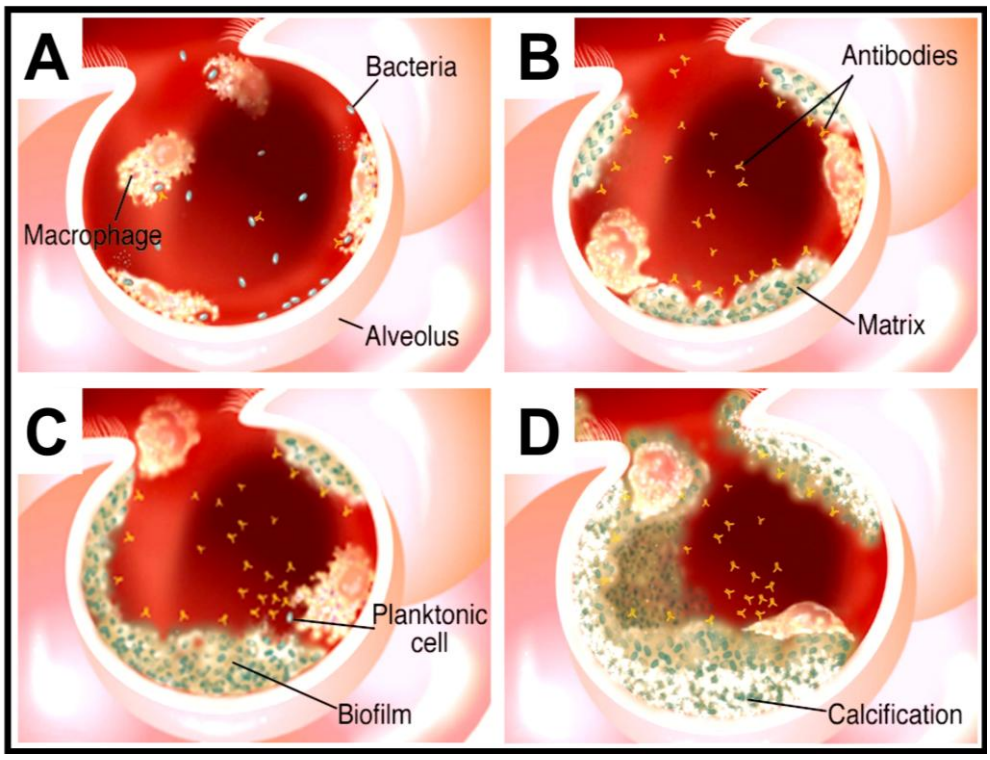

Figure 1.7 Development of a biofilm infection in the lung of Cystic Fibrosis. **A,** Surface of the alveolar epithelium is guarded by macrophages. **B,** Alveolar phagocytes are unable to engulf biofilm bacteria. **C,** Biofilm fragments grow in the colonized lung and release planktonic cells. **D,** The mature biofilm becomes calcified to form a long-term pulmonary nidus. Adapted from (Costerton *et al.*, 2003).

1.7 Biofilms and Antibiotic Resistance

One of the most important features of bacterial biofilms is their resistance to antimicrobial agents and the host immune system (Ciofu and Tolker-Nielsen, 2011).

Although antibiotics may decrease the number of bacteria in biofilms, they will not completely eliminate them which may have important clinical consequences such as persistence of infections that are biofilm-related (Ciofu and Tolker-Nielsen, 2011). It has been reported that biofilm bacteria are 1000 times more resistant to antibiotics than their free-living counterparts indicating that some of the mechanisms that mediates resistance varies between sessile and planktonic bacteria (Nickel *et al.*, 1985) which may lead to discrepancies between *in vitro* results and *in vivo* response (El-Azizi *et al.*, 2005). Therefore, shifting the mode of antibiotic regimens to include sessile bacteria rather than planktonic will improve the methods of treatment especially against biofilm-associated infections (Wolcott and Ehrlich, 2008). The resistance of biofilms to antibiotic treatment is often referred to as antibiotic tolerance, which is a physiological condition that allows the bacteria to survive in the presence of antibiotic concentrations above their planktonic minimal inhibitory concentration. (Ciofu and Tolker-Nielsen, 2011). The mechanisms that explain the antibiotic tolerance of biofilms can be categorised in the five groups.

1.7.1 Restricted Penetration of Antimicrobials

The bacteria within the biofilm structure are embedded in a matrix that includes exopolysaccharide (EPS), extracellular DNA and protein (Ciofu and Tolker-Nielsen, 2011). Effective antibiotics must cross the physical barrier of biofilm's matrix in order to reach the encased sessile bacteria and therefore eliminate the biofilm infection (Ciofu and Tolker-Nielsen, 2011). The increased synthesis of exopolysaccharide that will slow down and retard the penetration of antimicrobials is considered a successful mechanism for biofilms to develop tolerance (Cheema *et al.*, 1986). However, evidence suggests that restricted penetration of antibiotics through biofilms may occur in cases where the

antibiotic binds to components of the biofilm matrix itself (Ciofu and Tolker-Nielsen, 2011). Evidence suggested that oxygen limitation and low metabolic activity appear to be more important factors for the tolerance of biofilms (Walters *et al.*, 2003). However, delayed penetration might play a role in the tolerance to certain antibiotics such as aminoglycosides especially in Cystic Fibrosis (CF) lungs where antibiotic concentrations are too low to penetrate the EPS matrix (Ciofu and Tolker-Nielsen, 2011).

1.7.2 Differential Physiological Activity

The existence of bacterial populations with different physiological activities in biofilms appears to be a mechanism against environmental threats (Bjarnsholt *et al.*, 2009). Evidence suggests that differential physiological activity within the biofilm is caused by bacterial consumption of oxygen and nutrients leading to slow growth of sessile bacteria due to limitation in nutrients making them less susceptible to antimicrobial agents (Brown *et al.*, 1988). Previous studies showed that it is possible to kill majority of the cells in a biofilm by combined antimicrobials that can target different physiological subpopulations within a biofilm such as the combination of colistin and tetracycline against biofilms of *P. aeruginosa* PAO1 in flow-chambers (Pamp *et al.*, 2008). Another example is the combination of colistin and ciprofloxacin used against *P. aeruginosa* in CF patients (Hansen *et al.*, 2008).

1.7.3 Formation of Persister Cells

Another type of bacteria that participates in antibiotic tolerance of biofilms are the so-called persister cells (Brooun *et al.*, 2000; De Groote *et al.*, 2009). Persister cells are spore-like cells that may survive an antimicrobial challenge (Stewart and Dirckx, 2001a), they represent slowly or non-dividing bacteria that are less susceptible to

antibiotics than the bulk of the population. They are generally believed to be the result of a small subpopulation of bacteria that differentiates into a dormant state (Spoering and Lewis, 2001). These cells are thought to escape the activity of antibiotics that target fundamental cellular processes such as DNA replication, translation, and cell wall synthesis due to their reduced metabolism (Bjarnsholt, 2011; Spoering and Lewis, 2001). The pathways leading to the formation of persister cells are still unknown, however evidence suggested that bacterial toxin/antitoxin systems may play a role in *Escherichia coli* (Lewis, 2000). Moreover, it has been reported that fluctuations in the expression of toxin and antitoxin genes can lead to unbalanced toxin/antitoxin systems that allows the formation of persister subpopulations thus making it escape killing by antibiotics (Vazquez-Laslop *et al.*, 2006).

1.7.4 Specific Tolerance Factors

In addition to tolerance caused by the mechanisms described above, certain gene products that are produced specifically within biofilms may exhibit unique functions that can increase the antibiotic tolerance of the biofilm community (Ciofu and Tolker-Nielsen, 2011). An example of a biofilm-specific tolerance factor that occurs only in biofilms is quorum-sensing (QS) which has been reported to be involved in tolerance of *P. aeruginosa* biofilms to the antibiotics tobramycin and kanamycin in addition to hydrogen peroxide (Bjarnsholt *et al.*, 2005; Hassett *et al.*, 1999). Another example is the *ndv*B gene of *P. aeruginosa* PA14 which encodes an enzyme involved in the synthesis of periplasmic glucans that binds to the antibiotic tobramycin leading to prevent bacterial cell death and thus enhance tolerance (Mah *et al.*, 2003). A novel efflux pump of *P. aeruginosa* PA14 that is expressed only during the biofilm mode of growth and mediates resistance to tobramycin, gentamicin, and ciprofloxacin has been reported also as biofilm-specific tolerance factor (Zhang and Mah, 2008).

Because the presence of antibiotics is considered a harsh environment even for bacteria within biofilms, therefore other conventional resistance mechanisms that are not biofilm-specific will be also activated (Ciofu and Tolker-Nielsen, 2011). An example of non-biofilm specific tolerance factor are antibiotic degrading enzymes such beta lactamases which play a crucial role in the development of resistance of bacteria to beta-lactams (Bagge *et al.*, 2000). It has been reported that the presence of beta-lactamases in the biofilm matrix will lead to the hydrolysis of beta-lactam antibiotics before they reach the bacterial cells (Nichols *et al.*, 1989). The source of beta-lactamase in biofilms is thought to be from a sacrificial layer of bacteria that release these enzymes into the extracellular space when exposed to antibiotics (Ciofu and Tolker-Nielsen, 2011).

Another possible source of beta-lactamase in biofilms may be membrane vesicles containing beta-lactamase released by resistant bacteria (Ciofu *et al.*, 2000). Another example of non-biofilm-specific tolerance factors are the conventional efflux pump systems that can be up-regulated in response to certain triggers such as the up-regulation of efflux pumps during biofilm formation by *E. coli* isolates from urinary tract infection that helps in the removal of toxic substances and antibiotics (Kvist *et al.*, 2008). Has been suggested that potential use of efflux-pumps inhibitors and beta-lactamase inhibitors might improve the efficacy of antibiotic treatment against biofilm infections (Ciofu and Tolker-Nielsen, 2011). Other mechanisms that mediate biofilm tolerance include increased resistance to UV light (Bose and Ghosh, 2011), altered metabolism activity (Bose and Ghosh, 2011) and genetic exchange due to accumulation of genetic elements that facilitates horizontal gene transfer (Li *et al.*, 2002). Some of these mechanisms are illustrated in (Figure 1.8).

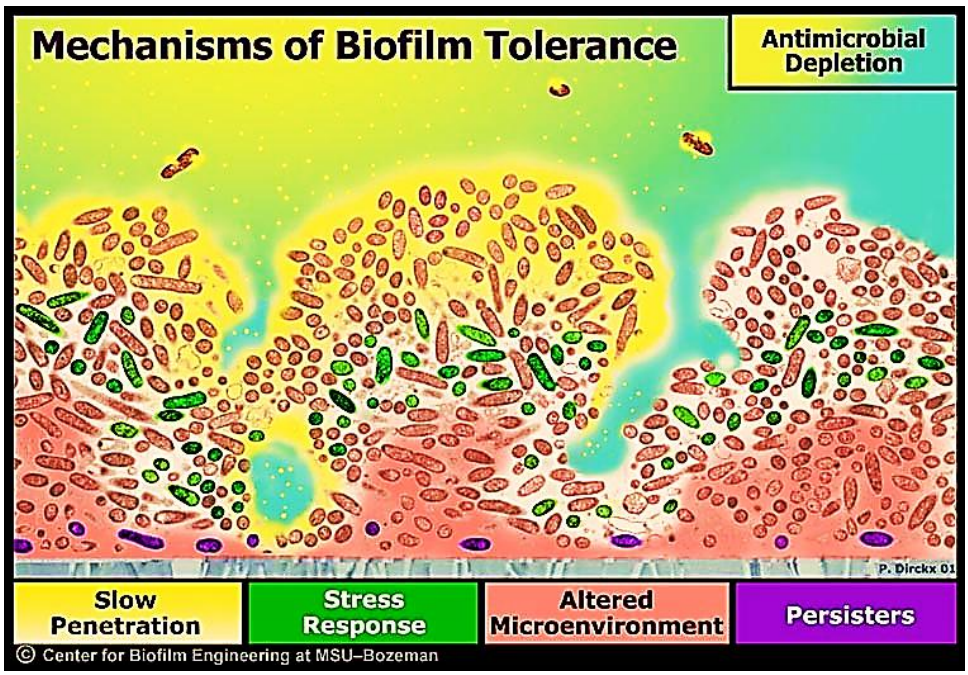

Figure 1.8 Mechanisms of biofilm tolerance to antibiotics. Adapted from (Stewart and Dirckx, 2001b).

1.8 Biofilms and Host Immune Responses

Since the early days of immunology, many studies have investigated the various mechanisms of immune responses to different microorganisms (Jensen *et al.*, 2010). However, the majority of these studies focused on studying the planktonic state of these infectious agents, therefore less is known about the immune responses to the presence of biofilm-associated infections. Nevertheless, more recent *in vivo* and *in vitro* studies have revealed both innate as well as adaptive immune responses to biofilms (Jensen *et al.*, 2010). The host immune system plays a key role in the clearance of bacterial infections (Fux *et al.*, 2003). However, persistent bacteria represent an important factor in the development of chronic infections (Høiby *et al.*, 1995). This persistence is mainly due to the formation of biofilm communities that possess an arsenal of resistance mechanisms to overcome the immune response particularly in immune compromised patients (Fux *et al.*, 2003).

The innate immune system is the first line of the defensive mechanisms in protecting the host from invading microbial pathogens (Akira *et al.*, 2001). It is designed to recognize a broad spectrum of foreign antigens present on numerous microorganisms (Moser and Jensen, 2011). The ability to detect invading microorganisms is accomplished by pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs) and signal their presence which will result in activation of host response (Jensen and Moser, 2011). Even though several classes of PRRs and their ligands are known, PRRs specific for biofilm-growing microorganisms have not yet been identified (Jensen and Moser, 2011). One of the examples of PRPs that have been intensively studied for its interaction with biofilms are the complement receptors. However the role of compliment receptors during biofilm infections is still not yet well established due to difficulties in establishing the presence of biofilms in clinical samples and the ability of biofilm bacteria to establish an infection in spite the activation of complement systems, therefore no cases of biofilm infections have been reported for patients with complement deficiency so far (Jensen and Moser, 2011). Another example of PRPs that is active against biofilm infections are the Toll-Like Receptors (TLRs) that are integral membrane glycoproteins with cytoplasmic domains transmitting intracellular signalling (Akira *et al.*, 2001). Evidence for the involvement of TLRs in biofilm infections has mainly come from chronically infected CF patients and from dental plaques (Jensen and Moser, 2011). Because the pathogen recognition capacity of neutrophils mostly rely on TLRs (Parker *et al.*, 2005), investigations of TLRs on the neutrophils of chronically infected CF patients have recently been highlighted (Jensen and Moser, 2011).

The adaptive immune response is characterized by a higher degree of specificity and memory in comparison with the innate response (Moser and Jensen, 2011). It recognizes species or even strain specific antigens, this memory is characterized by a

clonal expansion of specialized lymphocytes during the first exposure resulting in a significantly faster and stronger affinity response (Moser and Jensen, 2011). Activation of the adaptive immune response during a biofilm infection follows the same mechanisms as during an infection with the same microorganism during a non-biofilm forming infection. However, the effector mechanisms of the immune system against biofilm infections are insufficient in eliminating the pathogen. Therefore, the difference between the adaptive immune response to a biofilm and a non-biofilm infection lies in the impaired clearance of the microorganism that may contribute to the pathology of the infection itself (Hoiby *et al.*, 2001; Schaudinn *et al.*, 2009).

In biofilm infections, the persistent bacteria can resist the antibodies, phagocytes as well as other components of the host response and the surrounding tissue will be subject to oxidative radicals and enzymes released from the host itself (Moser and Jensen, 2011). In addition to various pathogen-specific virulence factors the release of proteases and other exoenzymes from the host cells can result in degradation of important surface molecules on the immune cells and thereby contribute to the impaired anti-biofilm effect of the host (Horvat and Parmely, 1988; McCormick *et al.*, 1997). An important feature of biofilm infections is the release of specific antigens by sessile cells (Cochrane *et al.*, 1988) that will stimulate the production of antibodies and cytokines by the host (Leid *et al.*, 2002). However, the difficulty of these antibodies to penetrate the biofilm's EPS can cause deposition of immune complexes and oxidative burst of macrophages that will damage the local host environment more than the biofilm itself (Høiby, *et al.*, 1995).

1.9 Examination of Biofilms

Biofilm formation is one of the known virulence factors of many pathogens and it is considered as a marker of virulence that can be detected phenotypically (Jain and Agarwal, 2009). Early detection and management of potentially pathogenic bacteria and their ability to form biofilms is essential to prevent biofilm-associated infections (Jain and Agarwal, 2009). There are a variety of *in vitro* and *in vivo* systems available to examine bacterial biofilms (Merritt *et al.*, 2011). Biofilm model systems are essential to gain a better understanding of the mechanisms involved in biofilm formation and resistance to both therapeutics as well as immune defences (Coenye and Nelis, 2010).

1.9.1 *In vitro* Biofilm Model Systems

Two main approaches are employed to study biofilm formation *in vitro*, static systems and chemostat or continuous-flow systems (Merritt *et al.*, 2011). Among the most frequently used systems is the Microtiter plate (MTP) assay were biofilms are grown on the bottom of a microtiter plate well and detected quantitatively (Christensen *et al.*, 1985; Shakeri *et al.*, 2007). This method was originally developed to study the attachment of coagulase-negative *Staphylococcus aureus* to plastic tissue culture plates (Christensen *et al.*, 1985), but has been since used to study many other bacterial species (Peterson *et al.*, 2011). Advantages of MTP assay include higher throughput, low cost, simplicity allowing less labour and no specialised equipment in addition to offer the opportunity to process large numbers of compounds and samples making it ideal for screening purposes (Niu and Gilbert, 2004) like screening for antimicrobial and anti-biofilm effects (Shakeri *et al.*, 2007) of various active compounds including antibiotics (Amorena *et al.*, 1999), disinfectants (Pitts *et al.*, 2003), chemicals such as quorum sensing inhibitors (QSI) (Brackman *et al.*, 2009) and medicinal plant extracts (Quave *et al.*, 2008). Another method for the detection of biofilm-forming pathogens is Congo

Red Agar (CRA) were the formation of biofilms is examined qualitatively (Jain and Agarwal, 2009). Other *in vitro* systems include flow displacement biofilm model, cell-culture-based model and microfluidic devices (Jain and Agarwal, 2009).

1.9.2 *In vivo* Biofilm Model Systems

There are different *in vivo* biofilm models that uses a variety of animals to mimic variety of diseases (Rumbaugh and Carty, 2011). The main advantage of using animal models in studying medical biofilms is to include the host defences and complex bodily fluids that are difficult to replicate *in vitro* (Rumbaugh and Carty, 2011). This is particularly important when studying the toxicity, bioavailability, clearance and absorbance effects of therapeutics on microorganisms in a biofilm (Rumbaugh and Carty, 2011). The most common approach that can reflect a typical human cause of infection were biofilms can be evaluated at different points, is to infect a vertebrate animal with an organism of choice (Rumbaugh and Carty, 2011). One major advantage of this approach is that it can use strains of organisms that lack virulence factors and determine the contribution of various genes in the biofilm formation (Nallapareddy *et al.*, 2006; Swords *et al.*, 2004). Another approach, which can be more reflective of the natural infection process of biofilms *in vivo*, is to allow the animal's normal microflora to initiate the infection (Leknes *et al.*, 2005). However, it has a limitation that there are major differences in the composition of the microflora between some animals and humans (Marsh, 1995). Therefore, great consideration must be given when choosing the bacterial and animal species and experimental models to use (Rumbaugh and Carty, 2011). Examples of *in vivo* biofilm models are periodontal models, foreign-body models and soft-tissue models (Rumbaugh and Carty, 2011). The advantages of using *in vivo* models of biofilm-associated soft-tissue infections are the wide range of indications that can be performed on a variety of animal species. However, detection of biofilms on host

tissue is challenging and the need for certain surgical skills can pose an obstacle in performing the experiment (Rumbaugh and Carty, 2011). Clinical indications for these models include endocarditis, otitis media, keratitis, sinusitis, wound infections and chronic lung infections (Rumbaugh and Carty, 2011).

1.9.3 Microscopic Methods for Imaging Biofilms

Microscopy allows the visualization of bacterial biofilms either on an artificial surface (Yanagihara *et al.*, 2000) or on the host tissue itself (Schaber *et al.*, 2007). Much of the early investigations to examine biofilms relied heavily on the use of light and electron microscopy (SEM) (Donlan and Costerton, 2002). Light Microscopy (LM) is the essential tool for biofilm observation either directly or through a histologic section (An and Friedman, 1997). The use of LM coupled with different stains such as Haematoxylin & Eosin (H&E) is one of the simplest most common methods to view the bacteria involved in an infection (Rumbaugh and Carty, 2011). However, a disadvantage to examine biofilms via LM is the difficulty in distinguishing bacterial cells from host tissue when using H&E due to its property in staining all nucleic acids (Rumbaugh and Carty, 2011). For higher magnification and better visualization of biofilm cells, researchers have used both Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) (Rumbaugh and Carty, 2011). The most frequent system is SEM were it can capture highly detailed images of the biofilm (Rumbaugh and Carty, 2011). However, a disadvantage is encountered when samples are fixed in a series of dehydration steps leading to reduce the total volume of biofilm's matrix and alter its architecture (Akiyama *et al.*, 2003).

For a fundamental understanding and proper examination of bacterial biofilms, detection must include both the bacteria and its glycocalyx matrix (Kania *et al.*, 2007). The most effective and non-destructive approach for studying biofilms in tissue samples

is via Confocal Laser Scanning Microscopy (CLSM) (Oliveira and Cunha Mde, 2010) coupled with a double staining technique (Kania *et al.*, 2007). This will allow the ability to visualize thick biofilm sections and observe the location of the microbial populations within the hydrated EPS, thus providing a more realistic image of the biofilm *in situ* (Rumbaugh and Carty, 2011).

1.10 Control of Biofilms

The emerging resistance of pathogenic bacteria to antibiotics remains a serious challenge to medicine and healthcare (Guo *et al.*, 2012). Examples of this challenge is the resistance of *Staphylococcus aureus* to methicillin (Moran *et al.*, 2006), the resistance of *Pseudomonas aeruginosa* and *Clostridium difficile* to fluoroqinolones (Hsu *et al.*, 2005; Loo *et al.*, 2005) and the resistance of *Salmonella* to multiple drugs (Chen *et al.*, 2004). The rate at which new antimicrobial agents are being discovered and approved doesn't match the rate at which the currently used antibiotics become less effective which makes it crucial to search for new antimicrobial agents that are active, safe and environmentally-friendly (Guo *et al.*, 2012). Current approaches to search for new antibiotics include metabolic engineering (Khosla and Keasling, 2003), synthetic chemistry (Piel, 2002), genomic or metagenomic approaches (Koehn and Carter, 2005; Poulsen *et al.*, 2011) and identifying new microorganisms for their ability to produce bioactive natural products (Jensen *et al.*, 2007). Traditional treatment of bacterial infections is based on compounds that kill or inhibit the growth of bacteria (Geddes, 2000). In the case of biofilms, evidence suggests that a single compound is not sufficient to eradicate the infection because such treatment will leave persistent bacteria to colonize neighbouring sites and will lead to the development of antibiotic resistance (Drenkard, 2003).

Recently, the pharmaceutical industry has acknowledged the importance of biofilm infections (Alhede *et al.*, 2011), however, there are still no commercially available antibiotics that can specifically target the biofilm mode of growth in addition to the increase resistance to available antibiotics (Boucher *et al.*, 2009). The development of biofilm specific drugs focuses on three major strategies which are prevention of biofilm formation, removal of biofilm and weakening of biofilm (Alhede *et al.*, 2011). Prevention of biofilm formation includes mechanisms such as the use of antibodies and pilicides (Alhede *et al.*, 2011). Whereas removal of biofilms include the use of bacteriophage (Sulakvelidze *et al.*, 2001), bacteriophage lytic enzymes (Fischetti, 2005), probiotics (Hong *et al.*, 2005), antimicrobial peptides (De Smet and Contreras, 2005), electrical currents (Del Pozo *et al.*, 2008), and dispersal signals (Davies and Marques, 2009). Moreover, weakening of biofilms includes the use of quorum sensing inhibitors (QSI), Inhibition of type III secretion and surface coatings (Alhede *et al.*, 2011). Example of antibiofilm strategies are the Polysaccharide Intercellular Adhesin (PIA) enzymes that are produced by the gene products of *ica*ADBC operon, making the *ica* genes a potential target for biofilm inhibition (Oduwole *et al.*, 2010). These enzymes have therapeutic benefits that can be used as biomaterial coating agent to reduce device-related infections (Oduwole *et al.*, 2010).

The development of substances that can specifically inhibit bacterial virulence such antipathogenic drugs are assumed to prevent the development of resistant strains thus inhibit biofilm formation (Bryers, 2008). A recent approach to combat such biofilms includes the blockage of regulatory systems that control the expression of virulence factors (Bryers, 2008) such as anti-adhesion therapy (Casadevall *et al.*, 2004), disrupting iron metabolism (Kaneko *et al.*, 2007), and up-regulating the promoters for biofilm detachment (Boles *et al.*, 2005).

1.11 Microbial Natural Products

The traditional definition of natural products is that they are chemical (carbon) compounds isolated from diverse living organisms and may derive from primary or rather secondary metabolism of living organisms (Berdy, 2005). The primary metabolites such as polysaccharides, proteins, nucleic and fatty acids are common in all biological systems. However, the secondary metabolites are chemically and taxonomically diverse compounds with various functions that are characteristic mainly to some specific type of organisms (Berdy, 2005). Natural products continue to play an important role in the discovery and development of new compounds during drug development (Lang *et al.*, 2008). An important part of the natural products is a group of small molecular secondary metabolites of microorganisms that usually exhibits biological activities (Berdy, 2005).

The natural environment remains a rich reservoir for microorganisms capable of producing potent antimicrobial agents (Clardy *et al.*, 2006). Many efforts worldwide focus on isolating natural products from terrestrial and marine macro- and microorganisms (Lang *et al.*, 2008) Screening for microbial natural products, i.e. bioactive secondary metabolites, represents an important step towards the discovery of novel therapeutic chemicals (Courtis *et al.*, 2003; Gillespie *et al.*, 2002) due to the fact that these natural products are the origin of most of the antibiotics in the market today (Pelaez, 2006). Many of these compounds are produced by the organism in response to extreme environments such as competition for space and nutrient in addition to potential predators (Proksch, 1994).

Although some secondary metabolites like extracellular polysaccharides (EPS) are synthesized by a wide range of bacteria and have been proven to be involved in pathogenicity (Campos *et al.*, 2004), recent findings suggested that some polysaccharides secreted from some bacteria inhabiting natural ecosystems possess the

ability to negatively regulate biofilm formation (Jiang *et al.*, 2011; Qin *et al.*, 2009). The advantages of natural products compared to their synthetic counterparts is that they are largely unexplored, have unmatched chemical diversity with biological potency (Verdine, 1996), are considered as powerful pathfinders in the investigations of cellular functions (Zhang, 2005), and can help in the design of synthetic compounds (Breinbauer *et al.*, 2002). In addition, the research on these products has led to the discovery of novel mechanisms of action (Urizar *et al.*, 2002).

1.11.1 Bioactive Metabolites of Marine Bacteria

Bacteria are one of the main producers of the antagonistic substances in terrestrial environment. Many activities have been reported from marine bacteria including antibiotic, antiviral, antifungal, and anti-yeast activities (Bhakuni and Rawat, 2005; Buck *et al.*, 1962). Examples of such marine bacteria include the ichthyotoxic *Pseudomonas piscicida* that exhibits a significant antagonism to various microorganisms, *Pseudomonas bromoutilis* that produces the antibiotic bromo pyrrole active against many Gram-positive bacteria (Lovell, 1966) and *Serratia marcescens* that produces a red coloured antibiotic named prodigiosin with antifungal and anticancer activities (Bennett and Bentley, 2000; Llagostera *et al.*, 2003). The formation of antibiotic substances from such bacteria indicates that marine microbes are capable of producing new and unusual types of antibiotic substances when compared to the terrestrial ones some of which will be useful in developing medicine and pharmaceuticals (Bhakuni and Rawat, 2005).

It has been shown that extracts of marine actinomycetes act as a quorum sensing inhibitor (QSI) exhibiting antibiofilm activity against *Vibrio harveyi* by attenuating the signal molecules N-acylated Homoserine Lactones (You *et al.*, 2007). Another marine bacteria with reported antibiofilm activity is *Bacillus pumilus* from Palk Bay that

inhibits the quorum-sensing-mediated virulence factors in Gram-negative bacteria (Nithya *et al.*, 2010a).

1.11.2 Bioactive Metabolites of Soil Bacteria

Despite the fact that soil is arguably the most useful and valuable habitat on earth, it is still considered one of the least understood ecosystems that needs to be further explored (Handelsman *et al.*, 1998). Evidence shows that less 0.1% of soil the microorganisms are readily cultured using current techniques (Torsvik *et al.*, 1990) and the other 99.9% are still emerging as vast collection of novel genetic diversity (Torsvik *et al.*, 1996). This great diversity will probably lead to further diversity even within species which current phylogenetic analysis cannot resolve (Handelsman *et al.*, 1998). Estimates are that a gram of soil might contain 1,000-10,000 species of unknown microorganisms (Torsvik *et al.*, 1990). Soil carries the highest concentrations of microbial populations when compared to any other habitat, it still remains an important source of microbial natural products (Whitman *et al.*, 1998).

In the past, a large number of soil organisms, mainly Actinomycetes and fungi, have been isolated and screened for valuable products (Vandamme, 1994). Bacteria on the other hand play a major role in the complex diversity of microorganisms in soil due to the fact that a large portion of them are still not identified (De Vos, 2011). New groups of bacteria have been identified in soil that appear to differ greatly from the cultured bacteria that they could represent new phyla or even new kingdoms of life (Borneman *et al.*, 1996). In addition to new natural products, novel soil bacteria are also of strategic importance in the discovery of new bioactive secondary metabolites (Saravanan *et al.*, 2012). Therefore, taxonomically novel soil bacteria are of strategic importance in the discovery of new bioactive secondary metabolites (Saravanan *et al.*, 2012).

1.11.3 Soil Bacterial Species of *Paenibacillus*

The genus *Paenibacillus* was first identified in 1993 based on a comparative analysis of 16S rRNA sequences of 51 species belonging to the genus *Bacillus* (Ash *et al.*, 1991; Ash *et al.*, 1993). The newly proposing genus of *Paenibacillus* comprised of aerobic or facultatively anaerobic rod-shaped, endospore-forming bacteria (Montes *et al.*, 2004). At that time, the genus comprised 11 species, with *P. polymyxa* as the type species (Seldin, 2011). To date, there are 150 species of *Paenibacillus* approved and validated according to the bacterial nomenclature list by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Braunschweig (DSMZ), Germany. (May 2013). This bacteria are harbouring strains of medical, industrial and agricultural importance (Seldin, 2011). In order to identify and analyse the properties of *Paenibacillus* from a mixed environment, preparation of a pure culture is essential (Bodour *et al.*, 2003). The microbe of interest can be cultivated, selected and enriched by using an enrichment culture that will provide favourable growth conditions for the organisms of interest and unfavourable for the competing organisms (Bento *et al.*, 2005; Sen, 2010; Willumsen and Karlson, 1997). In the past few years, the 16S ribosomal RNA (16S rRNA) gene sequence has become the gold standard method for accurate identification of microorganisms, as it relates to bacterial taxonomy and the discovery of novel bacteria (Sintchenko, 2010). This method is particularly important in the case of slow-growing bacteria, uncultivable bacteria and bacteria with unusual phenotypic profiles in which it will allow for the discovery of new bacterial species and genera (Clarridge, 2004; Hall *et al.*, 2003; Woo *et al.*, 2008).

Although some species of *Paenibacillus* like *P. larvae* cause disease in larval honeybees, no *Paenibacillus* species has been reported as a human pathogen (Govan *et al.*, 1999). Strains of *Paenibacillus* spp. produce various antimicrobial agents such as lantibiotics (He *et al.*, 2007), lipopeptides (Martin *et al.*, 2003), macrolides (Wu *et al.*,

2011), immunomodulators, antitumor agents (Rodrigues *et al.*, 2006), and non-ribosomally synthesized compounds that are active against a wide range of bacteria, fungi, and oomycetes (Ongena and Jacques, 2008).

Interest in *Paenibacillus* spp. as a source of new antimicrobials has been increasing (Guo *et al.*, 2012). They are widely distributed in the environment, particularly soil were some of them promote the growth of plant are involved in nitrogen fixing (Khianngam *et al.*, 2009; McSpadden Gardener, 2004; von der Weid *et al.*, 2002).

1.11.4 Identification of Microbial Natural Products

The bioactive metabolites produced by bacteria in natural environments could be a mixture of several class of chemical compounds (Bhakuni and Rawat, 2005). Chemically these compounds can be classified either as amino acids, peptides, nucleosides, alkaloids, terpenoids, sterols, saponins or polycyclic (Bhakuni and Rawat, 2005). Since the chemical nature of these compounds are unknown, it is not possible to follow any specific technique for the separation of the constituents of that complex mixture (Bhakuni and Rawat, 2005).

The process of separation involves the extraction of significant amounts of the desired organism to obtain the crude extracts mixture followed by separation of the mixture that can be achieved by fractionation with organic solvents. Each of these fractions is then subjected to biological assays in order to identify the possible bioactive compounds that can then be isolated by a bioassay-guided fractionation process (Lang *et al.*, 2008). If the separation is good, the biological activity may concentrate in a particular fraction (Bhakuni and Rawat, 2005) which will eventually lead to yield pure compounds that can be then characterized by various techniques such as Mass Spectrometry (MS), Ultraviolet (UV), Infrared (IR), and Nuclear Magnetic Resonance

(NMR) spectroscopy (Lang *et al.*, 2008). However, the therapeutic applications of some natural products has declined during the last decade due to certain limitations such as the competition from chemically-synthetic compounds as a source of new drugs (Pelaez, 2006), the redundancy of strains and compounds within natural-product libraries (Handelsman *et al.*, 1998), the lack of a systematic exploitation of natural ecosystems (Czaran *et al.*, 2002), the labour-intensive and time consuming approaches used in characterizing such active compounds (Monaghan *et al.*, 1995) and the need for an extensive media optimization and scale-up when producing large quantities for drug profiling (Strobel, 2002).

CHAPTER TWO

LITERATURE REVIEW

2.1 Prevalence of Biofilm Infections in Otorhinolaryngology

According to the United States, microbial infections that are associated with biofilms make up to 80% of all human infections (Stowe *et al.*, 2011) leading the U.S. Centres for Disease Control and Prevention (CDC) to declare biofilms as one of the most important medical problems of the century (Davies, 2003). Various studies had shown that chronic infections including the ear, nose and throat are associated with biofilms (Hall-Stoodley *et al.*, 2006; Kania *et al.*, 2007). Therefore, otorhinolaryngology as well as other medical disciplines are facing the challenge of biofilm-related infections (Vlastarakos *et al.*, 2007) which makes it crucial to develop novel strategies for the prevention and treatment of such infections (Vlastarakos *et al.*, 2007).

In a prospective study made by Psaltis *et al.*, they compared the presence of bacterial biofilms on the sinus mucosa of 38 Chronic Rhinosinusitis (CRS) patients and 9 control patients using CLSM. Bacterial biofilms were found in 44% of CRS patients whereas no biofilms were present in any of the controls which support the hypothesis that biofilms may play a role in the pathogenesis of CRS (Psaltis *et al.*, 2007). The study of recurrent acute otitis media (RAOM) presented by Zuliani *et al.*, pointed out the variety of biofilm-related infections noting that biofilms were present in the adenoids that were collected by his team stating that the degree to which biofilms are present in relation to controls is striking and should not be ignored (Zuliani *et al.*, 2006). Another study by Zulianai *et al.*, described by a comparative microanatomic investigation of adenoid mucosa using SEM in patients with recurrent acute otitis media (RAOM) and

obstructive sleep apnea (OSA) (27 female and 41 male; age range, 3 months to 15 years) (Zuliani *et al.*, 2009). The study showed evidence of bacterial biofilms covering the entire surface of adenoids removed from patients with RAOM with an average of 93.53% of their mucosal surface covered, versus an average of 1.01% coverage from patients with OSA (Zuliani *et al.*, 2009). A similar study was performed by Coticchia and co-workers, demonstrated that the adenoids removed from patients with CRS had dense mature biofilms covering 94.9% of their mucosal surface compared with 1.9% coverage on the adenoids removed from patients with OSA (Coticchia *et al.*, 2007). Moreover, the mechanical removal of these biofilms may explain the observed clinical benefit associated with adenoidectomy in this group of patients (Coticchia *et al.*, 2007).

Direct detection of biofilms on biopsy specimens from children with otitis media effusion (OMED) and recurrent otitis media supports the hypothesis that these chronic middle-ear disorders are biofilm-related (Cryer *et al.*, 2004). Ramadan *et al.*, demonstrated in their study that biofilms were present in patients undergoing surgery for CRS. However, none of the patients without CRS had any evidence of biofilms (Costerton *et al.*, 1999). Even though researchers had described new methods that effectively remove biofilms, studies did not prove that these biofilms actually caused the CRS and RAOM. Therefore, more investigations are needed to prove that biofilms are the causative agents for such conditions (Zuliani *et al.*, 2006). There is also evidence for the possible presence of bacterial biofilms on frontal sinus stents in patients with chronic sinusitis who underwent Functional Endoscopic Sinus Surgery (FESS) and these stents may actually serve as biofilm reservoirs (Perloff and Palmer, 2004).

Study by Chole *et al.*, examined the palatine tonsils that had been surgically removed due to infection and obstruction by SEM and TEM was found that bacteria were seen within the tonsillar crypts of 11 of 15 infected tonsils and in 3 of 4 hypertrophied tonsils (Chole and Faddis, 2003), which indicates that there is strong

anatomical evidence for the presence of bacterial biofilms in chronically diseased tonsils. Another study by Kania *et al.*, demonstrated the presence of mucosal biofilms in most 17 of 24 patients with tonsillitis (70.8%) by direct visualization of bacteria and glycocalyx using CLSM with double fluorescent staining on tonsils and compared the findings with the results of scanning electron microscopy analysis (Kania *et al.*, 2007). Al-Mazrou and Al-Khattaf studied the presence of bacterial biofilms on the epithelial surfaces of tonsils and adenoids in children undergoing adenotonsillectomy (T&A) (Al-Mazrou and Al-Khattaf, 2008) and their results showed adherent biofilm formation in 46 patients (61%). Among 26 patients with infections, adherent biofilm formation was detected in 22 (85%), whereas in the group of 44 patients with obstruction only 18 were found to have biofilms (41%).

A study by Winther *et al.*, used LM to determine the location of bacteria and their biofilm in the adenoid tissue and in the mucus overlying the adenoid (Winther *et al.*, 2009). They reported the bacterial biofilms were present on 8 of 9 adenoids and bacteria were not found in the parenchyma of the adenoids below the epithelial surface whereas planktonic bacteria were present in 7 adenoids in areas not subject to mucus flow (Winther *et al.*, 2009). Review by Lipton and Gozal provided a comprehensive analysis of the morbidity and treatment of OSA in children. However, the conclusion was that they were still unable to determine an appropriate cost-effective guidelines for treatment and therefore efforts to develop novel and more effective therapies should be taken into consideration (Lipton and Gozal, 2003).

2.2 Worldwide Antibiofilm Discoveries

There are several examples of naturally produced compounds that inhibit the process of biofilm formation, one class of which are the antagonists of quorum sensing

or anti-quorum sensing molecules (Maximilien *et al.*, 1998). The first observation came from the marine red alga *Delisea pulchra* which secretes N-acyl homoserine lactones (AHL) analogs, called furanones, and that their activity correlates with reduced surface colonization of the plant by bacteria (Maximilien *et al.*, 1998). Study by Hentzer *et al.*, demonstrated that a synthetic halogenated furanone compound is capable of interfering with the AHL-mediated QS system in *P. aeruginosa* and that furanone treatment destabilized biofilms making the treated bacteria sensitive to antibiotics while the untreated highly resistant, suggesting that it has potential to be used as a novel treatment for biofilm-associated infections (Hentzer *et al.*, 2002).

Recently, the metabolomics approach has been used to classify metabolites based on metabolite profiling studies (Kim *et al.*, 2009) allowing rapid analyses of complex data and the determination of novel compounds (Rochfort, 2005; Want *et al.*, 2005). In an effort to find viable sources of antibiofilm agents, many researchers have started to extract and analyse natural products from microorganism inhabiting natural ecosystems (Donia and Hamann, 2003). In a study by Ren *et al.*, it has be shown that the QS antagonist (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone) from the marine alga *Delisea pulchra* inhibits biofilm formation in *E. coli* without inhibiting its growth (Ren *et al.*, 2004). Another study showed that the metabolites of a marine *Actinomycete* strain A66 inhibit biofilm formation by *Vibrio* in marine ecosystem (You *et al.*, 2007). Thenmozhi *et al.*, demonstrated that extracts from coral associated *Bacillus horikoshii* inhibit biofilm formation of *Streptococcus pyogenes* (Thenmozhi *et al.*, 2009). Compounds having anti-biofilm activity and secreted from microorganisms living in natural environments like marine and soil ecosystem, can range from furanone to complex polysaccharide (Abu Sayem *et al.*, 2011). Recent findings suggest that some polysaccharides secreted from marine and non-marine

organisms also possess the ability to negatively regulate biofilm formation (Jiang *et al.*, 2011; Qin *et al.*, 2009).

Many studies used Liquid Chromatography-Mass Spectrometry (LC-MS) as a metabolite profiling tool which allows more comprehensive metabolite analyses (Bino *et al.*, 2004; Silas *et al.*, 2005) allowing high sensitivity, high-throughput and ability to identify components in complex samples (Krug *et al.*, 2008). A study by Kim *et al.*, presented an approach for the high-throughput screening of secondary metabolites from 4 bacterial strains of *Myxococcus xanthus* (Kim *et al.*, 2009). Their results detected 22 metabolite peaks of the bacterial crude extracts via Electrospray Ionization-Mass Spectrometry (ESI-MS) followed by metabolite profiling via m/z value, retention time, and MS/MS fragmentation pattern analyses. Among the peaks, one unknown compound peak was identified as analogous to the myxalamid A, B, C series and further analysis identified a new compound (Kim *et al.*, 2009).

Recent study by Moon *et al.*, examined the effect of FDA-approved iron chelators deferoxamine (DFO) and deferasirox (DFRA) against planktonic and biofilm cells of *Prevotella intermedia* in order to evaluate the possibility of the iron chelators as alternative controlling agents against *P. intermedia* (Moon *et al.*, 2013). Their results showed DFRA partially inhibited the bacterial growth and considerably prolonged the bacterial doubling time however DFO was unable to completely inhibit the bacterial growth in the concentration range tested. In addition, crystal violet binding assay for the assessment of biofilm formation by *P. intermedia* showed that DFRA significantly decreased the biofilm-forming activity as well as the biofilm formation, while DFO was less effective (Moon *et al.*, 2013).

Another recent study by Alves *et al.*, evaluated the *in vitro* effects of four natural substances of farnesol, xylitol, lactoferrin and salicylic acid on the biofilm mass formed by *Enterococcus faecalis* and *Staphylococcus epidermidis* via crystal violet assay to

assess their potential use as root canal irrigants (Alves *et al.*, 2013). Their results showed that combination of farnesol and xylitol was the most effective agent against *E. faecalis* biofilms. Similarly, combinations involving farnesol, xylitol, and lactoferrin reduced the biomass of *S. epidermidis* biofilms as well concluding that combinations of antibiofilm substances have potential use in endodontic treatment to combat biofilms (Alves *et al.*, 2013).

Another study by Payne *et al.*, involved screening of a library of small molecules for the ability to inhibit the biofilm formation of *Staphylococcus aureus*. The authors presented evidence that the polyphenolic compound tannic acid inhibits the biofilm formation of *S. aureus* in multiple biofilm models without inhibiting bacterial growth via a mechanism dependent upon the putative transglycosylase IsaA (Payne *et al.*, 2013). In addition, the same investigators developed a rodent model for *S. aureus* throat colonization and found that the consumption of tannin-containing drinks like tea reduced the colonization of *S. aureus* via an IsaA-dependent mechanism. These findings provide insight into a molecular mechanism by which polyphenolic compounds, such as tannins, influence the surface colonization of *S. aureus* and act as antibiofilm agents (Payne *et al.*, 2013).

A study by Liu *et al.*, used a collection of β-peptoid-peptide hybrid oligomers displaying different amino acid/peptoid compositions and chain lengths to study their antimicrobial activity against *Staphylococcus epidermidis* both in planktonic and biofilm cultures and compare their effects with those of the common antibiotic vancomycin via susceptibility tests, time-kill assays and microscopic observations (Liu *et al.*, 2013). Their results showed that all tested peptidomimetics were bactericidal against both exponentially growing and stationary-phase *S. epidermidis* cells with similar killing kinetics and that Chiral and guanidinylated hybrids exhibited the fastest killing effects against slow-growing cells and had more favourable antibiofilm

properties than analogues only containing lysine or lacking chirality in the β-peptoid residues. Concluding that this class of peptidomimetics may contain promising antimicrobial alternatives for the prevention and treatment of multidrug-resistant *S. epidermidis* infections. (Liu *et al.*, 2013).

2.3 Current Research of Biofilm in Malaysia

Many studies have been conducted in Malaysia to address the problems and ways to combat biofilms in various fields. In 2010, we have reported the presence of bacterial biofilms among clinical samples via an oral presentation at the annual scientific meeting paper awards of the Malaysian Society of Otorhinolaryngology Head and Neck Surgeons (MSO-HNS). We have reported and compared the identification of bacterial biofilms among patients undergoing tonsillectomy for chronic and recurrent tonsillitis using various microscopic techniques (Rahmat *et al.*, 2010).

In the same year, we have participated in the same year at the Innovation and creativity EXPO 2010 University of Malaya and reported a study entitled "Discovery of Novel Properties from Bacterial Culture Supernatant via A Rapid and Simple Method towards Developing Anti-Quorum Sensing and Anti-Biofilm Drugs" (Rahmat *et al.*, 2010). The novelty of this work lies in the discovery of an antibiofilm property from a novel soil bacterial culture filtrate as an environmentally-friendly naturally-produced compound against clinically important pathogens allowed us to win the gold medal award and the best category award (Appendixes 1A, 1B, 2A).

Moreover, we have participated in the 21st International Invention, Innovation & Technology Exhibition (ITEX 2010) held in Kuala Lumpur by presenting a study entitled "Biofilm profile of chronic and recurrent tonsillitis using various microscopic techniques and their outcome on the diagnosis and treatment of biofilm-related

infections" (Rahmat *et al.,* 2010) for which we have won the ITEX silver medal (Appendixes 1C, 2B).

Another study by us reported the potential activity of a cell-free supernatant isolated from a taxonomically novel bacterial species that was originally isolated from an agricultural soil. This study was presented as a poster presentation at the 15th Biological Science Graduate Congress/University of Malaya (Appendix 2C). We reported the *in vivo* activity of this compound in disrupting and breaking down the bacterial biofilm caused by *Pseudomonas aeruginosa* using a rat model of chronic lung infection (Saad *et al.,* 2010). Moreover, the bioactive property from the novel indigenous soil bacterial species was also reported and presented by us under the name soil-derived biocleaner at the Malaysian Technology Expo at Putra World Trade Centre and the Innovation and creativity EXPO, University of Malaya, Kuala Lumpur (Salmah *et al.,* 2010).

A study by Tan and Tay from University of Malaya described the killer phenotypes of tropical environmental yeasts and the inhibition effects of their culture filtrates on the biofilm of *Candida albicans* (Tan and Tay, 2011). Their study showed more than 50% reduction in the metabolic activity of *C. albicans* biofilm after exposure to the culture filtrates of the nine killer yeasts suggesting the purification and characterization of toxin and metabolites to be essential for understanding the killing effects of their selected yeasts (Tan and Tay, 2011). Another study from University of Malaya was conducted by Ramli *et al.* where they investigated the effects of environmental factors such as temperature, growth medium, pH and glucose on biofilm formation among 28 *Burkholderia pseudomallei* clinical isolates acquired from University Malaya Medical Centre (UMMC) and obtained from different sites of infection such as wounds, respiratory tract, urine, splenic biopsy, pus and blood in order to study the variations between different strains (Ramli *et al.,* 2012).

Study by Siddiqui *et al.* from Universiti Malaysia Pahang assessed the novel biological anti-quorum sensing (QS) of Piper beetle extract to control membrane biofouling in order to allow sustainable performance of membrane bioreactors for wastewater treatment (Siddiqui *et al.*, 2012). Their results demonstrated the occurrence of the N-acyl-homoserine-lactone (AHL) autoinducers, correlate QS activity and membrane biofouling mitigation indicating that extracts from the plant Piper beetle could be a novel agent to target autoinducers for mitigation of membrane biofouling (Siddiqui *et al.*, 2012).

Another study addressing the approach of QSIs was performed by Anbazhagan *et al.* from University of Malaya where they reported the detection of N-acyl-homoserine lactone (AHL) signalling molecules among 50 biofilm-forming clinical isolates of *Acinetobacter s*pp. obtained from University Malaya Medical Centre using the *Chromobacterium violaceum* CV026 biosensor monitor system (Anbazhagan *et al.*, 2012). Only two isolates of this species produced the AHL molecules which led them to conclude signal molecules aid in biofilm formation and pathogenicity of the clinical isolates including drug resistance (Anbazhagan *et al.*, 2012).

A study by Torey and Sasidharan from University Sains Malaysia was carried out to evaluate the inhibitory effect of methanol extract of *Cassia* (C.) *spectabilis* leaves against biofilm forming *Candida albicans* using disc diffusion assay and broth dilution method and examined via Scanning Electron Microscopy and Confocal Laser Scanning Microscopy (Torey and Sasidharan, 2011). Their results revealed that the methanol extract of *C. spectabilis* showed anti-yeast activity against *C. albicans* with a minimum inhibitory concentration (MIC) value of 6.25 mg/ml and that the main changes observed after exposure to the extract treatment were cellular damage and disruption in the biofilms of *C. albicans* indicating an *in vitro* antibiofilm activity of *C. spectabilis* leaves extract against Candida biofilms (Torey and Sasidharan, 2011).

A study by Odeyemi from the National University of Malaysia aimed to investigate biofilm production among *Vibrio* species isolated from Siloso Beach, Singapore where sea water samples were collected and plated on Congo Red Agar (CRA) and the biofilm producing colonies were further transferred into a selective medium for isolating *Vibrio* species. (Odeyemi, 2012). The results of the study revealed 46 biofilm positive bacteria were isolated after 24 h incubation on CRA concluding that open sea water for bathing or other recreational purposes could pose danger to human health and marine life in addition of being a vehicle of transmitting pathogens from one country to another especially if no disease or infection symptoms were noticed before departure of the infected tourist (Odeyemi, 2012).

A study by Nawawi *et al.* from Universiti Kebangsaan Malaysia addressed the microbially-influenced corrosion of metal surfaces by comparing the potential corrosion of 2 strains of marine sulfate reducing bacteria on stainless steel using electrochemical techniques (Nawawi *et al.*, 2012). The results revealed that differences of Rp value that were recorded between two bacterial strains mostly depend on the corrosiveness of the metabolomics substances produced and production rate of biofilm directly influenced by proportional with the bacteria growth (Nawawi *et al.*, 2012).

A study by Jassim *et al.*, from Universiti Putra Malaysia explored new approaches of phage-based bio-process of specifically pathogenic *Escherichia coli* bacteria in food products within a short period (Jassim *et al.*, 2012). Their results revealed that *E. coli* biofilms contaminating food products were reduced >3 log cycles upon using the phage cocktail of 140 highly lytic designed phages concluding proper prevention methods of pathogenic *E. coli* achieved in this study can replace the current chemically less effective decontamination techniques (Jassim *et al.*, 2012).

Another study from Universiti Putra Malaysia was conducted by Atshan *et al.* Their investigation aimed to demonstrate whether the characteristics of biofilm

formation are consistently similar among isolates variations of methicillin-resistant *Staphylococcus aureus* (MRSA) (Atshan *et al.*, 2012). The methods used to evaluate these characteristics were a modified Congo red agar (CRA) assay, a microtiter plate (MTP) assay, Light Microscopy (LM), Scanning Electron Microscopy (SEM) and the Polymerase Chain Reaction (PCR). Their results indicated that differences in biofilm production capacities are caused by the differences in surface protein A (Spa) type and are not due to differences in MLST and SCCmec types (Atshan *et al.*, 2012).

A study by Ibrahim *et al.* from Universiti Teknologi Malaysia was conducted to characterise and study the microbial flocs formed from raw textile wastewater in a prototype Aerobic Biofilm Reactor system for their potential use in the treatment of textile wastewater (Ibrahim *et al.*, 2009). Their results revealed that after 90-100 days of operation, microbial flocs of loose irregular structures were obtained from the reactor with good settling velocity and sludge volume index and that the molecular analysis of the flocs via 16S rRNA sequencing showed 98% homology to those of *Bacillus* sp., *Paenibacillus* sp. and *Acromobacter* sp. The authors concluded that when the flocs were used for the treatment of raw textile wastewater, they showed good removal activity indicating their potential application (Ibrahim *et al.*, 2009).

A study by Shunmugaperumal and Ramamurthy from the International Medical University in Kuala Lumpur was carried out to assess the anti-adherent and antibiofilm activities of Magnesium fluoride MgF(2) nanoparticles-stabilized oil-in-water nanosized emulsion over glass coupons against pathogenic microorganisms like *Escherichia coli* and *Staphylococcus aureus* (Shunmugaperumal and Ramamurthy, 2012). The results showed massive biofilm formation on non-emulsion-coated surfaces while emulsion-coated surfaces dramatically restricted bacterial colonization suggesting that MgF(2) nanoparticles-stabilized emulsion is effective in restraining bacterial colonization on glass surfaces and that increased antibiofilm action would be expected when these

nanoparticles are coated on other types of biomaterial surfaces such as intraocular lenses and catheters (Shunmugaperumal and Ramamurthy, 2012).

2.4 Importance of this Study

It has been observed that many ENT infections are becoming less susceptible to common treatment and more related to the presence of biofilms (Nixon and Bingham, 2006). Many studies had shown that mucosa-related ENT chronic infections are biofilm-related such as the study conducted by Palmer, which has identified the presence of biofilms in sinonasal mucosa and on stents removed after frontal sinus surgery from human patients (Palmer, 2006). Another investigation was conducted to study the relation between chronic inflammation of the tonsils, clinical features, and the presence of biofilms in the crypts in patients presenting with obstructive hypertrophy and recurrent upper airway pathology (Diaz *et al.*, 2011). Their results showed that all patients had tonsillar hypertrophy (Grade III or IV), for which 77.28% of tonsils harboured biofilms in their crypts demonstrating that symptoms like harsh raucous sound, tonsillar and adenoid hypertrophy, apnea, and cervical adenopathies are clearly related to the presence of biofilm in tonsils. This finding enabled them to propose that biofilms are involved in the pathogenesis of tonsils and adenoids hypertrophy (Diaz *et al.*, 2011)

However, studies on the presence of bacterial biofilms on lymphatic tissue surfaces are still lacking (Kania *et al.*, 2007). Therefore, we have selected the palatine tonsil as our clinical specimen because it is the only easily accessible human lymphoid tissue which often is considered as an example for lymphoid organs (Nave *et al.*, 2001), its remarkable role in the immunological defence of the body (Mogoanta *et al.*, 2008), tonsillitis is one of the most common infectious diseases of the upper respiratory tract

and that tonsillectomy is the most frequent surgical procedures performed worldwide (Kania *et al.*, 2007). In addition, the presence of biofilms in the tonsils has been reported mainly using tools that might have destructive effects on the integrity of biofilm structure such as SEM and TEM (Chole and Faddis, 2003; Parsek and Singh, 2003) which emphasizes the importance of visualizing and studying biofilms using a non-destructive approach like CLSM.

Failure of antibiotics to eradicate the pathogenic bacteria that are responsible for otorhinolaryngologic diseases has led to the hypothesis that these bacteria may be presented in a biofilm structure (Drago *et al.*, 2012). A recent study was conducted to evaluate the ability of bacterial isolates recovered from tonsillar biopsies and swabs od pediatric patients to form biofilms *in vitro*. The ability of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* to form biofilms was evaluated spectrophotometry via MTP assay (Drago *et al.*, 2012). The results showed that 44.7% of the intraoperative samples were either moderate or strong biofilm producers compared with 27% of isolates at 6 months after surgery concluding that the ability to form biofilm decreased in bacteria isolated after tonsillectomy which suggests a role for biofilm in pathogenesis of recurrent and chronic pharyngeal diseases such as tonsillitis (Drago *et al.*, 2012). Therefore, in our study, we collected the palatine tonsils from patients undergoing elective tonsillectomy and/or adenoidectomy for various tonsillar diseases due to infection and/or obstruction, then we detected the presence of biofilms microscopically using the three main microscopic tools LM, SEM and CLSM and correlate the microscopic finding with the clinical manifestations of tonsillar diseases that are due to infection and/or obstruction. This was followed by an assessment to the ability of tonsillar bacterial isolates to form biofilm by means of spectrophotometry in order to test the hypothesis that chronic and recurrent tonsillitis are biofilm-related.

Important properties of biofilm-growing bacteria are different from those of planktonic bacteria and this has significant diagnostic and therapeutic consequences (Hoiby *et al.*, 2010). In clinical specimens such as biopsies and swabs, biofilm can be visualized by light microscopy, however, a more precise identification of bacteria within biofilm can only be done by DNA hybridization techniques (Hoiby *et al.*, 2010), and identification of the components of biofilm's matrix require specialized staining techniques such double staining (Bjarnsholt *et al.*, 2009). Traditional culturing techniques may not be sufficient to culture bacteria in the biofilm mode of growth because tit reveals only the properties of planktonic bacteria. Therefore, antibiotic susceptibility testing based on routine culturing might give misleading results that do not reflect the increased resistance of the bacteria living in biofilms (Hoiby *et al.*, 2010).

Many studies have reported that biofilm-growing bacteria are more resistant (up to 1000-fold) when compared with planktonic bacteria (Anwar and Costerton, 1990; Bjarnsholt *et al.*, 2007; Moskowitz *et al.*, 2004) and various methods have been developed to test the susceptibility of biofilm bacteria to antibiotic (Anwar *et al.*, 1990). However, the clinical relevance of these methods with regard to prediction of clinically successful therapies is still not confirmed (Anwar *et al.*, 1990; Moskowitz *et al.*, 2004).

It has been reported that the antibiotics used to treat pulmonary infections like CF are typically chosen based on the results of antimicrobial susceptibility testing of bacteria in a planktonic mode of growth (Waters and Ratjen, 2012). Moreover, evidences suggested that *Pseudomonas aeruginosa* grows in biofilms in the airways of CF, therefore, choosing antibiotics based on biofilm rather than conventional antimicrobial susceptibility testing could potentially improve response to treatment of CF infections (Waters and Ratjen, 2012). Therefore, in our study we blindly assessed the antimicrobial susceptibility of the tonsillar bacterial isolates by conventional

culturing methods and correlate it with the ability of these isolates to form biofilms qualitatively and quantitatively.

The failure of currently available antibiotics in controlling biofilm-associated infections makes it crucial to find alternative drugs (Hentzer *et al.*, 2003). The administration of antimicrobial agents and biocides has been shown to be useful in combating biofilms (Danese, 2002). However, prolonged persistence of these compounds in the environment could induce toxicity towards non-target organisms and develop resistance among microorganisms (Abu Sayem *et al.*, 2011). This aspect has led to the development of more environmentally-friendly bioactive compounds that can target a wider range of bacterial infections (Abu Sayem *et al.*, 2011).

It has been reported that the genus *Paenibacillus* has been isolated from various ecological habitats, including warm springs (Chou *et al.*, 2007; Saha *et al.*, 2005), alkaline soils (Yoon *et al.*, 2005) and agricultural soil (Ma and Chen, 2008). Various properties were reported among *Paenibacillus* species including nitrogen fixation (Berge *et al.*, 2002), production of hydrolytic enzymes (Hrabák and Martínek, 2007) and antimicrobial substances (Mavingui and Heulin, 1994). However, According to DSMZ and to the best of our knowledge, there is no haemolytic activity reported for any *Paenibacillus* species (Salmah *et al.*, 2012).

Therefore in our study, we tested, purified and characterize the bacterial culture filtrate from a taxonomically novel species of *Paenibacillus* isolated from an agricultural soil for its antibiofilm activity *in vitro* and *in vivo* against clinically important pathogens recovered from tonsillar specimens in order to provide theoretical evidence on the prevention and treatment of biofilm-associated infections. It is hoped that with the finding of compound(s) with such activity we will contribute in the new therapeutic management of biofilm-associated infections such as chronic and recurrent tonsillitis.

2.5 Objectives of this Study

The aim of our study was to test the hypothesis that chronic and recurrent tonsillitis are biofilm-related and a compound from novel soil bacterial species of *Paenibacillus* strain 139SI exhibits antibiofilm activity against clinically important pathogens towards new therapeutic management of biofilm-associated infections. Advances in chemical profile analysis such as High Performance Liquid Chromatography (Zhang, 2005) and chemical structure characterization such as Liquid Chromatography-Mass Spectrometry (Guo *et al.*, 2012) has improved the efficiency of the discovery of novel antimicrobials from natural sources (Pelaez, 2006). Therefore, these analytical tools have been applied as key methods in our search for new bioactive metabolites of *Paenibacillus* sp. strain 139SI isolated from a Malaysian agricultural soil. This particular strain attracted our attention due to its remarkable haemolytic activity *in vitro* and the fact that the genus *Paenibacillus* is a rich source of compounds with potential therapeutic value. Therefore, the following objectives were assessed:

1. To detect the presence of biofilms in the palatine tonsils among patients undergoing tonsillectomy for chronic and recurrent tonsillitis.
2. To identify the microorganisms isolated from tonsillar specimens and assess their antimicrobial susceptibility and biofilm formation ability.
3. To analyse and purify the bioactive compounds from a novel soil bacterial culture filtrate and assess its toxicity and antibiofilm activity *in vitro* and *in vivo*.
4. To establish an animal model of chronic lung infection treated with compounds from novel soil bacterial culture filtrate and evaluate its histopathological effects.
5. To characterize and identify the potential antibiofilm compounds from soil bacterial culture filtrate.

CHAPTER THREE

MATERIALS AND METHODS

A. MATERIALS

3.1 Clinical Specimens

The clinical specimens in our study were collected from a total number of 70 patients undergoing elective tonsillectomy and/or tonsillectomy & adenoidectomy (T&A) at University Malaya Medical Centre (UMMC). From each patient, 2 surgically removed tonsils (left and right side) and 2 surface swabs were collected. Thus, the total number of clinical specimens was 280 comprising of 140 tonsillar biopsies and 140 tonsillar swabs.

3.2 Bacterial Isolates

Bacterial isolates in our study were represented by clinical isolates for which a total number of 484 bacterial isolates (302 Gram-Positive and 162 Gram-Negative) were recovered from tonsillar swabs and biopsies. Control reference strains for microtiter plate (MTP) assay were represented by biofilm forming strain for Gram-positive *Staphylococcus aureus* (ATCC 25923), biofilm forming strain for Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and non-biofilm forming strain *Escherichia coli* (ATCC 25922) (Revdiwala *et al.*, 2011)

A taxonomically novel bacterial strain namely *Paenibacillus haemolyticus* strain 139SI was originally isolated from an agricultural soil in Serdang Agricultural Centre (Pusat Pertanian Serdang) Selangor, Malaysia. It was identified via 16S rRNA test and

deposited at the American Type Culture Collection (ATCC) with a cataloguing number ATCC–BAA-2268 (Salmah *et al.*, 2012)

3.3 Chemicals, Reagents, Solutions and Consumables

The chemicals used in our study were absolute ethanol, xylene, acetone, ammonium formate, formic acid, acetonitrile, sucrose and teepol solution. The buffers used were phosphate-buffered saline (PBS) tablets and normal saline (0.9% NaCl). The compound 2(5*H*)-Furanone and sodium alginate were used in the chronic lung infection model.

The microbiology consumables included dehydrated culture media for the cultivation and growth of bacterial isolates were represented by Brain Heart Infusion Agar, Brain Heart Infusion Broth and Tryptic Soy Broth (BD, France). Columbia Agar with 5% Sheep Blood and Chocolate II Agar was obtained from ISOLAB, Malaysia. Plasticware consumables included inoculating loops volume of 10µl (Jet Bio-Fil), sterile conical centrifuge tubes capacity of 15 ml and 50 ml (Jet Bio-Fil), micro centrifuge tube capacity of 1.5ml (Eppendorf), disposable Petri dish, sterile, 90 mm x 15 mm (Thermo-Line) and square weight trays polystyrene. For the *in vitro* assays, we used tissue culture plates with different growth surface areas of 6 and 96 wells (Jet Bio-Fil) and disposable pipette tips with various volumes (Jet Bio-Fil). Glassware consumables included bijou bottles with aluminium screw cap and a capacity of 7ml (S Murray & Co), measuring cylinder, screw cap with different capacities (Schott Duran, Germany) and Erlenmeyer flasks narrow neck. For sterilization of soil bacterial supernatant, Minisart® cellulose acetate (CA) membrane syringe filters were used with a pore size of 0.22 µm and a diameter of 47 mm (Sartorius, Germany).

Histology consumables included specimen containers, fixative solution of 4% glutaraldehyde, scalpels, forceps, dissection boards, tissue cassettes, metal base molds, sectioning blades, biodisposer, slide boxes and paraffin wax. The mounting media Dibutyl Phthalate Xylene (DPX) was used in routine histology and as for immunohistochemistry staining (IHC), a special mounting medium was prepared with 9 parts of glycerol and 1 part phosphate buffered saline (PBS) followed by the addition of a small amount of an anti-quenching agent, i.e. *p*-phenylenediamine. Staining solution for routine light microscope were Gram stain, H&E stain, whereas for IHC staining under CLSM were the double stain of Concanavalin A (Con A) and Propidium iodide.

3.4 Experimental Animals

For our experimental study, a total number of 84 Sprague-Dawley (SD) rats were randomly selected including 36 rats (18 males + 18 females) for the acute toxicity test and 48 rats (24 males + 24 females) for the chronic lung infection model. All animals obtained from Animal Care Unit Centre (ACUC), Faculty of Medicine, University of Malaya were 6-8 weeks old and weighed between 150-180 g. Food and water was available daily except for the short fasting period prior to dosage administration of the tested soil bacterial filtrate. All animals were kept in the animal experimental room for at least 3 days prior to dosing to allow for their acclimatization to the conditions of experiments. The room was well ventilated and the lighting was regulated to reproduce a 12 h cycle of day and night with a temperature maintained at around 25°C.

3.5 Antimicrobial Agents

The selected antimicrobial agents that were used in this study to determine the antimicrobial susceptibility of bacterial isolates recovered from tonsillar specimens are listed in (Table 3.1).

Table 3.1 List of selected antimicrobial agents used in this study.

Generic Name (β-lactams)	Abbreviation Name	Antimicrobial Class	Disk Content	Zone Diameter Breakpoints
Ampicillin	AM	Penicillins	10 µg	S ≥ 24 – R ≤
Amoxicillin-Clavulanic Acid	AMC	β-lactamase inhibitor combinations	20/10 µg	S ≥ 20 – R ≤ 19
Ampicillin-Sulbactam	SAM	β-lactamase inhibitor combinations	10/10 µg	S ≥ 15 – R ≤ 11
Cefoperazone	CFP	Cephems (parenteral)	75 µg	S ≥ 21 – R ≤ 15
Cefotaxime	CTX	Cephems (parenteral)	30 µg	S ≥ 26 – R ≤ 22
Ceftazidime	CAZ	Cephems (parenteral)	30 µg	S ≥ 21 – R ≤ 17
Ceftriaxone	CTR	Cephems (parenteral)	30 µg	S ≥ 26 – R –
Cefuroxime	CXM	Cephems (parenteral)	30 µg	S ≥ 24 – R ≤ –
Cephalexin	LEX	Cephems (oral)	30 µg	S ≥ – – R ≤
Imipenem	IPM	Penems	10 µg	S ≥ – – R ≤
Methicillin	ME	Penicillins	5 µg	S ≥ 14 – R ≤ 9
Penicillin	PEN	Penicillins	10 µg	S ≥ 24 – R ≤
Piperacillin-Tazobactam	TZP	β-lactamase inhibitor combinations	100/10 µg	S ≥ 21 – R ≤ 17
Generic Name (Non-β-lactams)	**Abbreviation Name**	**Antimicrobial Class**	**Disk Content**	**Zone Diameter Breakpoints**
Amikacin	AN	Aminoglycosides	30 µg	S ≥ 17 – R ≤ 14
Azithromycin	AZM	Macrolides	15 µg	S ≥ 12 – R –
Clindamycin	CM	Lincosamides	2 µg	S ≥ 21 – R ≤ 14
Ciprofloxacin	CIP	Quinolones	5 µg	S ≥ 21 – R ≤ 15
Co-trimoxazole	SXT	Folate pathway inhibitors	1.25/23.75 µg	S ≥ 16 – R ≤ 10
Erythromycin	EM	Macrolides	15 µg	S ≥ 23 – R ≤ 13
Fusidic Acid	FA	Fusidane	10 µg	S ≥22 – R ≤19
Gentamicin	GM	Aminoglycosides	10 µg	S ≥ 15 – R ≤ 12
Rifampin	RA	Ansamycins	5 µg	S ≥ 20 – R ≤ 16
Vancomycin	VA	Glycopeptides	30 µg	S ≥ 17 – R ≤ –

B. METHODS

3.6 Selection of Patients

A total number of 70 patients diagnosed with different tonsillar diseases undergoing elective tonsillectomy and/or tonsillectomy & adenoidectomy (T&A) at UMMC were enrolled in this study. The duration for selecting patients and collecting their clinical specimens was 10 months starting from October 26, 2009 to July 26, 2010. Approval was obtained from UMMC medical ethics committee [PPUM/UPP/300/02/02Ref. No. 744.11] (Appendixes 4A, 4B, 4C).

Prior to surgery, patients who satisfied the inclusion criteria (Table 3.2) were selected and enrolled in the study. They were presented at the Otorhinolaryngology clinic and the ENT ward at UMMC with signs and symptoms of chronic and recurrent tonsillitis in addition to obstructive sleep apnea. The nature of the clinical research in terms of methodology, possible adverse effects and complications (as per patient information sheet) were explained directly to each adult patient and to a responsible relative for each pediatric patient. After knowing and understanding all the possible advantages and disadvantages of our clinical research, the patients who agreed voluntarily consent of their own free will signed the consent form given to them and participate in our study. The English and Malay versions of consent forms by responsible relative for our study can be found in (Appendixes 5A, 5B).

Table 3.2 Inclusion and exclusion criteria for selecting patients in our study.

Inclusion Criteria
1. Patients undergoing tonsillectomy due to 3 attacks of chronic or recurrent tonsillitis per year or 5 attacks in 2 years with symptoms like fever, snoring during attacks and inability to take normal diet. [a]
2. Patients undergoing tonsillectomy due to OSA with symptoms like nocturnal snoring with mild upper airway obstruction, complete cessation of airflow with gas exchange abnormalities and severe sleep disturbance. [b]
Exclusion Criteria
1. Patients with a history of an infectious illness and who received antimicrobial therapy within one month prior to surgery.[c]
2. Patients with grossly asymmetrical tonsillar size (unilateral hypertrophy) as noted on pre-operative clinical assessment. [c]
3. Patients undergoing tonsillectomy for emergency conditions such as acute airway obstruction, peritonsillar abscess and/or other deep neck space infections. [c]
4. Patients undergoing tonsillectomy for suspected benign or malignant tumors. [c]
5. Patients with OSA not due to adenotonsillar hypertrophy but to other causes such as craniofacial anomalies and neurologic abnormalities. [d]
6. Patients with type I and II diabetes mellitus and immunocompromised patients. [e]

[a] (Darrow and Siemens, 2002); [b] (Gozal, 2000); [c] (Kuhn *et al.*, 1995); [d] (Greenfeld *et al.*, 2003); [e] (Loganathan *et al.*, 2006).

3.7 Collection of Tonsillar Specimens

Prior to surgery, the Clinical Diagnostic Laboratory (CDL) was informed and the technicians stood by to receive the clinical specimens upon collection from the operating room. Patients underwent general anaesthesia followed by retraction of the uvula and soft palate. At that time, the inner surface of the two palatine tonsils was swabbed with a sterile cotton applicator for each side followed by the surgical removal of palatine tonsils via blunt dissection method (Loganathan *et al.*, 2006). As soon as the

tonsils were removed, they were aseptically cut into 4 pieces with a sterile surgical blade and were aseptically put into sterile and labeled containers (Al Ahmary *et al.*, 2012) and transferred immediately to CDL for processing.

3.8 Processing of Tonsillar Specimens

Upon collecting tonsillar biopsies, specimens were dissected into four parts, fixed with an appropriate fixative and then processed accordingly. The first tissue part was placed unfixed in a sterile container with no fixative and sent, along with the swabs, to the CDL at UMMC for proper isolation and biochemical identification of microorganisms. The second tissue part was placed unfixed in a sterile container to be processed freshly for immunohistochemistry and viewed via CLSM. The third tissue part was fixed with a cold 4% Glutaraldehyde, a common fixative for electron microscopy (Westra *et al.*, 2003), and further dissected into 1-mm cubes to be viewed via SEM. The fourth tissue part was fixed with 10% neutral buffered formalin, a standard fixative used in most laboratories (Westra *et al.*, 2003), to be processed for a histopathology examination at the Department of Pathology, Faculty of Medicine, University of Malaya.

3.9 Isolation and Identification of Tonsillar Bacterial Isolates

The time between removal of the tonsils and inoculation of the specimen did not exceed 20 min. As soon as the tonsillar specimens reached the CDL, biopsies were aseptically weighed then dipped in alcohol, air-dried for 5 min and flamed to remove surface contamination. Biopsies were placed in thioglycollate broth (THIO), which represents an enriched liquid medium for the growth of microaerophilic and anaerobic

bacteria, with a volume equivalent to 1:10 dilution on a weight/volume basis (1 ml/gm), they then were mechanically homogenized. Serial dilutions of 1:10 and 1:100 were accomplished in THIO and each dilution was poured in a nutrient media of blood agar. After incubation for 18 to 24 hours, the level of bacterial growth (bacterial load) was identified by colony counting (Sasaki and Koss, 1978). Bacterial identification was accomplished by routine culturing techniques on selective media such as chocolate agar (CHOC), MacConkey agar, fastidious anaerobic agar (FAA) and Kligler Iron Agar (KIA) in addition to various biochemical tests specific for each bacterial species (e.g. DNAse test for *S. aureus*; Optochin test, Bile Solubility test, and Bacitracin tests for *Streptococcus* spp.; Indole, Citrate, Malonate utilization, Urease, Oxidase, Methyl Red (MR) and Voges-Proskauer (VP) tests for *Enterobacteriacea* Gram-Negative Bacilli (GNB); and XV factor test for *Haemophilus* spp.).

3.10 Antimicrobial Susceptibility via Disk Diffusion Test

The most common method of antibiotic susceptibility testing used in clinical laboratories is the disk diffusion method (Pierce-Hendry and Dennis, 2010). The test was blindly assessed by the technicians at Clinical Diagnostic Laboratories, UMMC according to the protocols described previously (Clinical and Laboratory Standards Institute, CLSI, 2009b). Briefly, a fixed volume of nutrient broth containing a standard concentration of bacteria was smeared evenly onto the surface of Mueller-Hinton agar plate (MHA) and disks of filter paper each impregnated with a standard concentration of an antibiotic were applied to the plate surface followed by aerobic incubation. Each antibiotic diffuses at a different rate, achieving different concentrations in the surrounding agar based on its molecular size and chemical properties (Clinical and Laboratory Standards Institute, CLSI, 2009b).

The zones of inhibition were measured in millimetres and the edge of these zones correlated with the antibiotic concentration that inhibits the growth of the bacteria were compared to a standard table of pre-determined zone widths. These widths represent the antibiotic concentrations in the agar that correlate with the concentration of antibiotic achievable in the plasma of a patient using a manufacture's recommended dosage (Clinical and Laboratory Standards Institute, CLSI, 2009a). Based on the diameter of the inhibition zone and the CLSI interpretative criteria, the results were assigned to three categories, susceptible, intermediate, or resistant (Clinical and Laboratory Standards Institute, CLSI, 2009b). The raw data for all isolates was obtained for further analysis.

3.11 Microscopic Detection of Biofilm Presence

3.11.1 Scanning Electron Microscopy (SEM)

The protocol for visualizing biofilms in tonsillar tissue specimens using SEM was described previously (Kania *et al.*, 2007). Tonsillar biopsies were fixed in a cold 4% Glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C on a rotary shaker. The samples were then dehydrated through a graded series of acetone solutions (70%, 80%, 90%, 96%, and 100% acetone) for 20 min at room temperature, and critical point drying was performed. The specimens were then orientated, mounted on metal stubs, and sputter coated with gold (Polaron 5000; Polaron Equipment Ltd, Watford, Hertfordshire, England) before imaging. Specimens were examined using SEM (INCA x-sight, Oxford instruments, United Kingdom) with digital imaging capabilities available at the electron microscope unit at the Faculty of Medicine, University of Malaya. The images were collected at an acceleration voltage of approximately 5.0 kV, a filament current of approximately 10−10A, and a working

distance of approximately 39 mm. All images were digitized as high resolution TIFF computer files and were then converted to high-quality JPEG files using commercially available software (Kania *et al.*, 2007).

3.11.2 Confocal Laser Scanning Microscopy (CLSM)

The processing of tonsillar tissue specimens for the purpose of visualizing biofilms via CLSM was done as described previously (Kania *et al.*, 2007). To preserve the biofilm architecture, tonsillar biopsies were embedded in an optimum cutting temperature medium (OCT) and were immediately snap-frozen in a mixture of cold isopenthane and liquid nitrogen and stored at –80^{o}C. The frozen tissue specimens were cut into a thickness of 10 μm at –24°C using a cryostat (Leica Microsystems, Bensheim, Germany) and were fixed in 70% cold acetone for 10 min. The obtained sections were then processed for double staining for which they were washed 3 times with phosphate-buffered saline (PBS) solution and each slide was first stained with 500 μl Propidium iodide for 5 min at room temperature to detect the bacterial cells in red, followed by staining with 500 μl of Concanavalin A (Con A) fluorescent isothiocyanate (FITC) for 5 min at room temperature to detect the surrounding glycocalyx matrix in green.

Sections were then washed in a combined solution of phosphate-buffered saline (PBS) and de-mineralized water and were embedded in a mounting medium of PBS/Glycerol mix treated with *p*-phenylenediamine as an anti-quenching agent. The tissue sections were examined using CLM (LSM 700. Carl Zeiss, Germany) available at Universiti Putra Malaysia (UPM). The microscope was equipped with a krypton-argon laser for visualization of Con A FITC (number of signals acquired, 488 nm; emission 552 disconnecting filter [DF], 32 nm) and Propidium iodide (number of signals acquired, 568 nm; emission 605 DF, 32 nm) (Kania *et al.*, 2007). Digital images of the

CLSM optical sections were collected using the ZEN 2010 software then converted to high-quality JPEG files using commercially available software.

3.12 Biofilm Formation Ability

3.12.1 Quantitative Detection via MTP Assay

To test the biofilm formation ability of clinical isolates, we used a quantitative adherence assay as previously described (Baldassarri *et al.*, 2001; Baldassarri *et al.*, 2006). Briefly, a 1:10 dilution of overnight cultures in Tryptic Soy Broth (TSB) was used to inoculate wells in a 96-well microtiter polystyrene plate. After growth at 37°C for 18 h, plates were gently washed three times with PBS to remove free-floating bacteria. Bacterial attachment on the bottom of each well was fixed with sodium acetate (2%) followed by air drying at 60°C for 1 h before staining with 125 µl of (0.1% w/v) Hucker's crystal violet solution 10 min at room temperature. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. The optical density (OD) of stained adherent bacteria was determined with an automatic spectrophotometer (Novapath microplate reader; Bio-Rad Laboratories, Inc., CA) at wavelength of 570 nm (OD_{570}). These OD values were considered as an index of bacteria adhering to a surface and forming a biofilm (Snoussi *et al.*, 2008).

To compensate for possible differences in growth rates under the different incubation conditions and for strains with different characteristics, the adherence index (AI) was adjusted as an estimate of the density of biofilm which would be generated by a culture with an (OD_{600}) of 0.5. Experiment was performed in triplicate and the data was then averaged and standard deviation was calculated. Calculation of the A was done according to the following formula:

Adherence Index = mean density of biofilm (OD_{570}) × 0.5/mean growth (OD_{600}).

3.12.2 Qualitative Detection via CRA Method

The ability of tonsillar clinical isolates to produce slime and therefore form biofilms was detected qualitatively by CRA method as described by the original method developed previously (Freeman *et al.*, 1989). The media was prepared by adding 0.8 g of Congo red stain and 36 g of saccharose into 1000 ml of BHI agar. The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 min then added when the agar had cooled to 55°C. Clinical isolates were cultured on CRA plates and incubated for 24-48 h at 37°C (Snoussi *et al.*, 2008) followed by 2 days at room temperature (Deighton *et al.*, 1992). The Congo red dye will directly interact with certain polysaccharides, forming colored complexes (Jain and Agarwal, 2009).

For an accurate assessment of all the possible chromatic variations exhibited by the cultured colonies, a reference eight-color scale was adopted to accurately determine all the chromatic variations exhibited by the cultured bacterial colonies. The scale ranged from very black to very red and the colorimetric reference scale to evaluate the chromatic variations exhibited by the cultured bacterial colonies based on their ability to form slime is shown (Table 3.3). Biofilm forming strains were categorized as: very black, black and almost black colonies and were all considered as slime producers, whereas non-biofilm forming strains were categorized as: very red, red and bordeaux colored colonies.

Table 3.3 Colorimetric reference scale of bacterial colonies on Congo Red Agar

Color (Symbol)	Interpretation	Strain indication	Mark
1. Very Black (VB) [a, c]	Strong slime-producer	Biofilm former	+++
2. Bright Black (BB) [b]	Strong slime-producer	Biofilm former	+++
3. Opaque Black (OB) [b]	Moderate slime-producer	Biofilm former	++
4. Almost Black (AB) [a]	Weak slime-producer	Biofilm former	+
5. Bordeaux (BR) [a, b]	Non-slime-producer	Non-Biofilm former	-
6. Orange (OR) [c]	Non-slime-producer	Non-Biofilm former	-
7. Pinkish Red (PR) [c, d *]	Non-slime-producer	Non-Biofilm former	-
8. Very Red (VR) [a, c]	Non-slime-producer	Non-Biofilm former	-

[a] (Arciola *et al.*, 2002); [b] (Oliveira and Cunha Mde, 2010); [c] (Snoussi *et al.*, 2008); [d] (Deighton *et al.*, 1992); [*] Pinkish Red (PR) colonies usually appear smooth with a darkening at the center.

3.13 Preparation of Soil Bacterial Strain 139SI

3.13.1 Gram Staining and Morphology Confirmation

The most fundamental phenotypic characterization of bacteria is achieved by using Gram stain. A smear of soil bacterial culture was prepared from an overnight culture of *Paenibacillus haemolyticus* strain 139SI grown in an enriched media of BHI broth (BD laboratories). A suspension of strain 139SI was smeared onto a clean glass slide then fixed with heat and stained according to the procedure of Hucker's modification.

Culturing bacteria on blood agar is often considered as a preliminary screening method for the ability of those microorganisms to produce biosurfactants on hydrophilic media (Plaza *et al.*, 2006; Schulz *et al.*, 1991). Therefore, the bacterial culture was streaked onto Colombia agar supplemented with 5% sheep blood (ISOLAC®, ISOLAB Malaysia) and plates were incubated for 16–24 h at 37°C to ensure of its haemolytic

reproducibility. Strong haemolytic (β-hemolysis) colonies obtained from the plates were further observed for colony and cellular morphology, and Gram staining confirmation. The 139SI strains was routinely subcultured and maintained in a glycerol suspension (25%, w/v) at −80°C and BHI-slant agar at room temperature.

The cellular morphology of strain 139SI was observed under scanning electron microscopy using an overnight culture of strains SI grown in BHI broth. To prepare the sample for scanning electron microscope, 1 ml of pure bacterial culture was transferred into a sterile filter paper (1 ×1 cm) and air dried for 10 min. The sample was rinsed in 0.1 M phosphate buffer, followed by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature for 4 h. The cells were dehydrated by using series of ethanol concentrations: 30%, 50%, 70%, 85%, 95% and 100%, and then two times with 100% acetone. Critical point drying was done by Hitachi HCP-2 critical point dryer. The samples were then mounted on specimen stubs and sputter-coated with gold. The samples were observed by Zeiss DSM 950 scanning electron microscopy at 15 kV (Ko *et al.*, 2007).

3.13.2 Bacterial Culture Filtrate of Strain 139SI

In order to identify and analyze the properties of the isolate, preparation of a pure culture is essential and required (Bodour *et al.*, 2003). This is achieved by using an enrichment media that can provide favorable growth conditions for the organisms of interest (Bento *et al.*, 2005; Sen, 2010; Willumsen and Karlson, 1997). Therefore, an overnight culture of strain 139SI was transferred into 50 ml centrifuge tube under sterile conductions and the culture was centrifuged at 8000 rpm at 4°C for 20 min to separate the cell from the supernatant. The obtained supernatant was subjected to sterile filtration to which is considered the optimal method to remove all particles and dead microorganisms without any influence of their ingredients, therefore we used syringe

filter with a pore size of 0.22 µm for that purpose (Minisart® SARTORIUS, Germany) to yield a purified cell-free supernatant (CFS) to be assayed later for its antibiofilm activity.

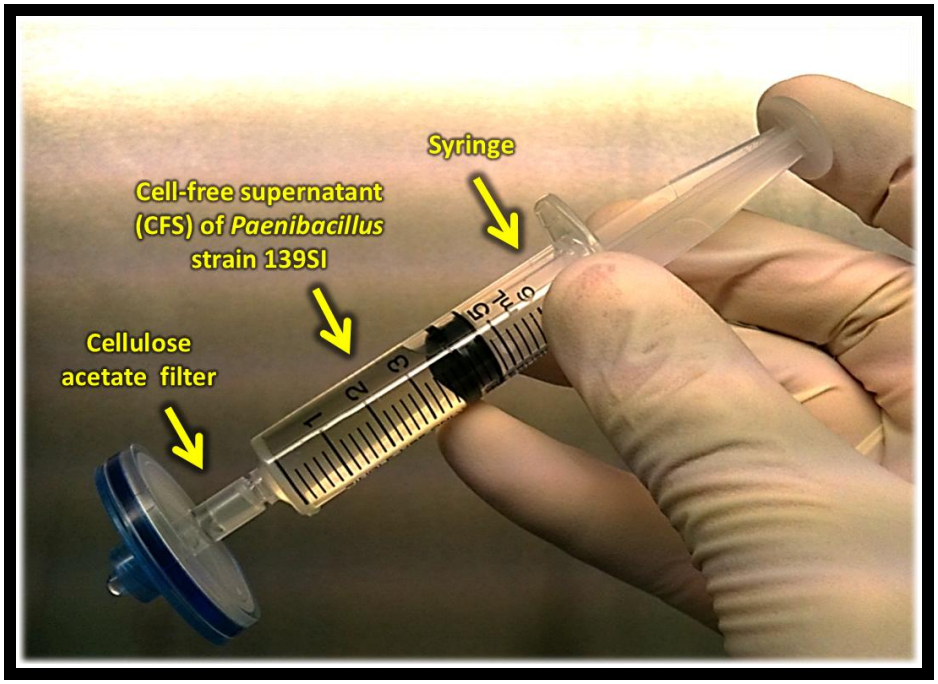

Figure 3.1 Preparation of bacterial culture filtrate from strain 139SI.

3.14 *In vitro* Antibiofilm Activity of 139SI Culture Filtrate

3.14.1 Determination of Biofilm Inhibition via MTP Assay

To determine the effect of the soil bacterial filtrate against the biofilm forming tonsillar isolates, MTP assay was performed as described previously by Baldassari *et al.* (2006) and Nithya, *et al.* (2010b) with few modifications as described by Kalishwaralal et al. (2010). Briefly, in each well of the 96-well microtitter plate, we transferred 180 µl of sterile BHI broth and inoculated it with 150 µl overnight culture of selected bacterial isolates followed by addition of 150 µl of soil bacterial culture filtrate with various concentrations (1000-4500 µg/ml). Plates were incubated and wells were stained

followed by measurement of the optical densities (Baldassarri *et al.*, 2006; Kalishwaralal *et al.*, 2010).

The selected Gram-positive isolates were *Staphylococcus aureus*, *Streptococcus agalactiae*, Group G Streptococci, *Streptococcus pyogenes* and *Streptococcus pneumoniae* whereas Gram-negative isolates were *Haemophilus influenza*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Citrobacter sp.* and *Pseudomonas aeruginosa*. A schematic representation of MTP assay is illustrated showing the concentrations used against selected clinical isolates (Figure 3.2).

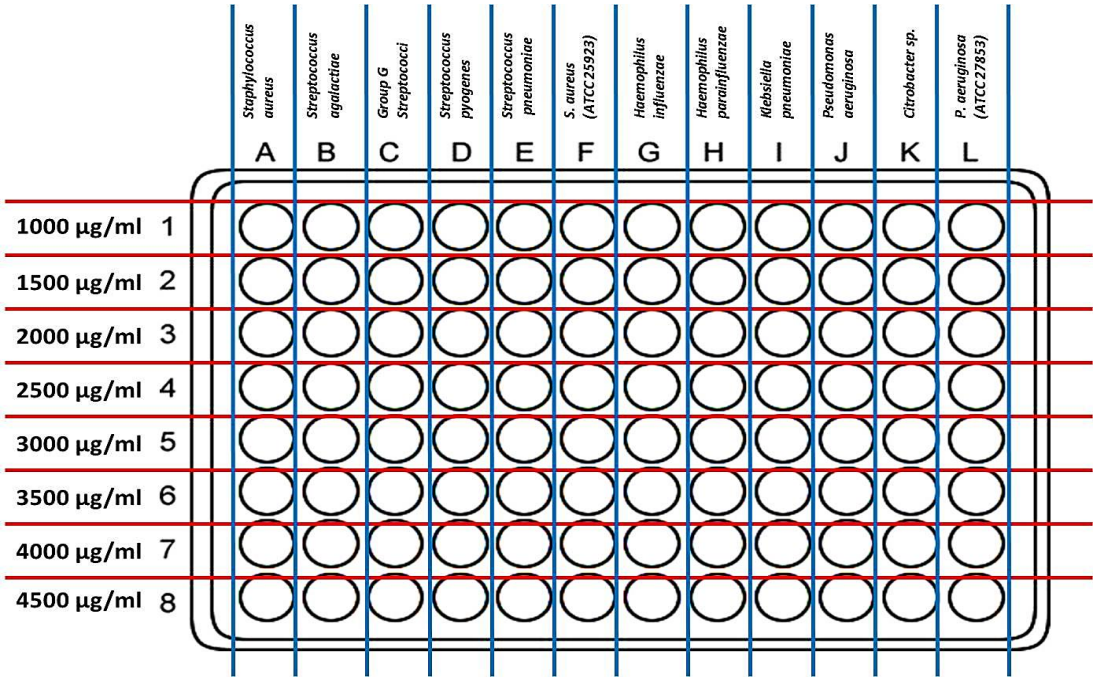

Figure 3.2 MPT assay of 139SI filtrate against selected clinical isolates.

3.14.2 Determination of Biofilm Inhibitory Concentration

In order to determine the lowest concentration of soil bacterial filtrate that can cause visible inhibition in the biofilm formation and significant reduction in the readings when compared with the control wells, the Biofilm Inhibitory Concentration (BIC) test was performed as described previously (Baldassarri *et al.*, 2006; Nithya *et al.*,

2010b) with few modifications as described by Kalishwaralal *et al.* (2010). The tonsillar bacterial isolate that was selected for the BIC test was a resistant *Pseudomonas aeruginosa* isolate recovered from one of our selected patients diagnosed with chronic tonsillitis, this isolate will be used later as the challenge strain to establish the animal model of chronic lung infection.

In each well, a piece of glass cover slip (1 ×1 cm) was placed inside each of the 6 wells to allow the growth and visualization of the filtrate effect on the tested bacterial isolate. We transferred 300 µl of sterile BHI broth into each well of the MTP and inoculated it with 160 µl overnight culture of *P. aeruginosa* followed by addition of 160ml of various concentrations of soil bacterial filtrate (1000-5000 µg/ml). Plates were incubated for 24 h at 37^{o}C and the biofilm inhibition was determined spectrophotometrically via MTP reader and microscopically via SEM (Baldassarri *et al.*, 2006; Nithya *et al.*, 2010a). The positive control used was synthetic 2(5H)-Furanone and the negative control was BHI broth media.

3.15 Microscopic Observation of Biofilm Inhibition

3.15.1 Light Microscopy (LM)

For visualization of the inhibitory effect of soil bacterial culture effect on *Pseudomonas aeruginosa* biofilms, light microscopy was used to visualize the glass pieces (1×1 cm) placed inside the 6-well plates that were treated with our filtrate as described previously (Nithya *et al.*, 2010b). After rinsing the free floating culture, the glass pieces were stained with crystal violet and were inspected by light microscopy at magnification of 10×, 40× and 100×. Visible biofilms were documented with an attached digital camera (Nikon, Eclipse, Ti 100).

3.15.2 Scanning Electron Microscopy (SEM)

For visualization of the inhibitory effect of soil bacterial culture effect on *P. aeruginosa* biofilms, scanning electron microscope was used to visualize the bottom of each of the 6-wells that were holding the same glass pieces described previously (1×1 cm) (Nithya *et al.*, 2010b). The bottom of the wells were cut and separated from the MTP then were first fixed with 0.1 M cacodylate-buffered 2.5% glutaraldehyde containing 0.075% (w/v) ruthenium red and 75 mM lysine for 20 min at room temperature. This step was followed by fixation with the same solution but without lysine for 2 h and finally fixed with 1% OsO_4 plus ruthenium red for an additional hour. Samples were dehydrated through a graded series of ethanol. Critical point drying and gold coating was performed then examination by SEM (INCA x-sight, Oxford instruments) (Baldassarri *et al.*, 2001; Nithya *et al.*, 2010a).

3.16 Acute Toxicity Test of 139SI Culture Filtrate

The novel isolate of *Paenibacillus* species strain 139SI has been tested previously in our laboratory via LD50 test to determine its virulence in experimental mice. It was found that none of the mice were affected by the bacterial strain, therefore it was used as a non-virulent control strain (Mahsa, 2013). In order to determine the safety (non-toxic) dosage of 139SI culture filtrate, acute toxicity test was carried out.

A total number of 36 Sprague Dawley (SD) rats (18 males and 18 females) were used for the acute toxicity test for which all surviving experimental and control group animals were sacrificed on day 15 for evaluation of the gross general observations, haematological profiles, biochemical parameters of blood and histopathology of liver, kidney and lungs (Akhand *et al.,* 2010). The soil bacterial culture filtrate was converted into powder by freeze-drying (lyophilizaton) and normal saline was used as a solvent

for the lyophilized extract. The control group was used to determine whether the normal saline causes any effects when compared with the untreated control (Johnson and Besselsen, 2002). However, the acute toxicity group was used to determine the rational treatment of the toxicity manifestations of new compounds and for the development of safer potential drugs (Al-Bari *et al.,* 2006). Two main doses were decided in the test represented by a low dose (2 gm/kg) equivalent to (0.36 gm/180 gm of body weight) and a high does (4 gm/kg) equivalent to (0.72 gm/180 gm of body weight). Evaluation of the toxicological effect for the two doses was carried out. The flow chart of acute toxicity test of our soil bacterial culture filtrate is illustrated (Figure 3.3).

3.16.1 Gross General Observation

Throughout the course of experiment, monitoring of animals before and after the administration of soil bacterial filtrate was carried out by observing for signs of toxicity, morbidity and mortality in addition to the behavioural signs such as food intake, salivation, muscular weakness, reflexes, piloerection, respiration (dyspnea), convulsion and any changes in locomotion such as whether the animals tend to stay quietly or actively moving in their cage as described previously (Akhand *et al.*, 2010). The body weight of each rat of both groups was taken before the administration of the soil bacterial culture filtrate and just prior to sacrifice them.

3.16.2 Biochemical Parameters and Haematological Profile

The animals were fasted overnight prior to necropsy and blood collection and were anasethized with an intra-muscular (IM) combination of Ketamine and Xylazine (1 ml of 100 mg/ml Xylazine with 9 ml of 100 mg/ml Ketamine) given at a dose of (0.1 ml/100g) body weight. Blood was drawn from the jugular vein under anesthesia and

samples were collected and immediately send to the CDL at UMMC to be assayed for haematological profile and biochemical parameters (Akhand *et al.*, 2010).

For biochemical parameters of serum, blood was collected into yellow caped VACUETTE® clot activator tube and renal function tests were assessed including sodium, potassium chloride, carbon dioxide, anion gap, urea and creatinine. In addition to liver function tests including total protein, albumin, globulin, total bilirubin, conjugated bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and G-glutamyltransferase. For haematological profile of whole blood, it was collected into violet caped VACUETTE® EDTA tubes and the total count (TC) of RBC and WBC, differential count (DC) of WBC and platelet count were assessed.

3.16.3 Gross Necropsy and Histopathology

All surviving animals were anesthetized intra-muscularly by a combination of Ketamine and Xylazine and quick sacrificing was performed by exsanguinations of jugular vein for blood sample collection. Gross postmortem examinations are performed on all terminated animals as described previously (Akhand *et al.*, 2010). After sacrifice, the thoracic cavity was opened by an excision through the peritoneum that was extended through the sternum and into the neck taking care not to puncture the heart or lungs. The rib cage was fully opened and the liver, kidney, and spleen were harvested then fixed with 10% neutral buffered formalin (NBF) for 1 day then sliced into pieces and fixed again with NBF for 2 days. Fixed tissues were then processed then stained with H&E stain and mounted on glass slides with diphenyl xylene (DPX) and observed under light microscope.

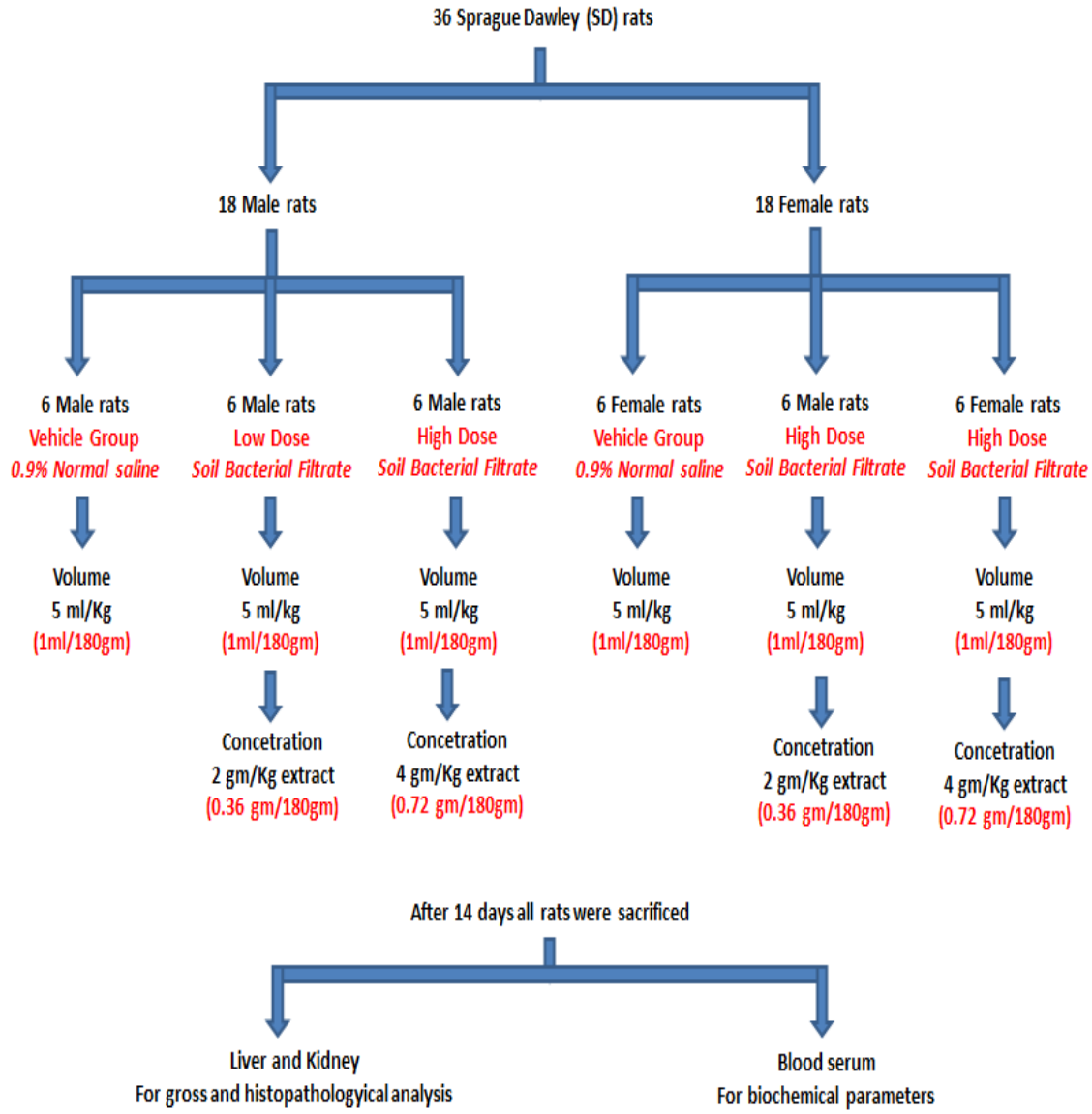

Figure 3.3 Flow chart of acute toxicity test of 139SI bacterial culture filtrate.

3.17 *In vivo* Antibiofilm Activity of 139SI Culture Filtrate

3.17.1 Preparation of the Infecting (Challenge) Strain

The challenge strain to establish a chronic lung infection was the multidrug resistant strain of *Pseudomonas aeruginosa* (ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, gentamicin and amikacin). This strain was isolated from

tonsillar biopsy specimen of a patient diagnosed with chronic tonsillitis. The isolate was embedded in an artificial alginate salt to mimic the condition of a biofilm chronic infection in the lung. Immobilization of the challenge strain was carried out as described previously (Pedersen *et al.*, 1990). The strain was incubated in 100 ml BHI broth for 24 hours at 37^{o}C and the culture was adjusted to yield 10^{9} CFU/ml followed by a modification whereby 1 ml of final culture were mixed with 9 ml of 1% sterile sodium alginate (Yan *et al.*, 2008). The purpose of adding alginate is to mimic the biofilm environment once it is inoculated into the lungs. The suspension was stored in a refrigerator at 4^{o}C until being used via intratracheal challenge to establish the animal model of chronic lung infection.

3.17.2 Establishment of Animal Model of Chronic Lung Infection

The duration of chronic lung infection model was 14 days and all groups except for the negative control were subjected to intratracheal challenge with 1 ml of challenge *P. aeruginosa* strain at day 1 and 3 (Yan *et al.*, 2008). The model comprised of the following groups: The first group represents the negative control where no changes from the normal state are expected to ensure that an unknown variable is not adversely affecting the animals in the experiment, which might result in a false-positive conclusion (Johnson and Besselsen, 2002). The second group represents the positive control that acts as a standard against which to measure difference in severity among experimental groups. It is expected to produce changes and to demonstrate that a response can be detected, thereby providing a quality control on the experimental groups (Johnson and Besselsen, 2002). The third group is the comparative control which is often a positive control with a known treatment that is used for a direct comparison to a different treatment (Johnson and Besselsen, 2002).

The fourth group was the experimental test group in which treatment effects of our soil bacterial culture filtrate were evaluated through the gross and histopathological observations of harvested lungs. This group was subjected to an oral administration with our soil bacterial culture filtrate at the same concentration, i.e. 4500 µg/ml, which showed *in vitro* activity via BIC test. The treatment was applied daily for each rat at a low dose of 30 mg/kg from day 1 until the sacrifice on day 15. Experimental design of the animal model of chronic lung infection is illustrated (Figure 3.3).

3.17.3 Lung Index of Macroscopic Pathology

The qualitative analysis of macroscopic lung pathology included abscess, consolidation, atelectasis and hemorrhage was expressed as the Lung Index of Macroscopic Pathology (LIMP), which was calculated by dividing the area of the left lung showing pathologic changes by the total area of the same lung (Song *et al.*, 1998). In addition, the gross pathological changes in the lungs were also assigned four different scores according to the severity of the inflammation previously described (Johansen *et al.*, 1994; Johansen *et al.*, 1993). The scores were I. Normal lungs; II. Swollen lungs, hyperemia, and small atelectasis (<10 mm^2); III. pleural adhesions and atelectasis (<40 mm^2); and IV, abscesses, large atelectasis, and hemorrhages (Yan *et al.*, 2008).

3.17.4 Histopathology Scoring of the Lungs

The lung pathology was assigned microscopically as described previously (Yan *et al.*, 2008). One of four scores according to the severity of the inflammation were set to assess the histopathological effects. These include the following scores:

1. Normal histology.
2. Mild focal inflammation.
3. Moderate to severe focal inflammation with areas of normal lung tissue.

4. Severe inflammation to necrosis or severe inflammation throughout the lung.

The cellular alterations were classified as acute or chronic inflammation by a scoring system based on the proportions of neutrophils (PMNs) and mononuclear leukocytes (MNs) in the inflammatory foci. Acute inflammation was defined as an inflammatory infiltration in which PMNs were predominant (≥90% PMNs with ≤0% MNs), whereas chronic inflammation was defined as a preponderance of MNs (≥90% with ≤10% PMNs), which included lymphocytes and plasma cells, and the presence of granulomas (Johansen *et al.*, 1994; Song *et al.*, 1998). The lung tissue with pathological changes were fixed, embedded in paraffin wax and sectioned prior to staining with both H&E stain and double stain to evaluate the severity of microscopic pathology by light microscopy and CLSM, respectively (Wu *et al.*, 2004). In order to confirm the cellular morphology of the infecting *Pseudomonas aeruginosa* isolate embedded in alginate within the infected alveoli, lung specimens were processed for SEM as described in session 3.11.1 and colony morphology on Nutrient Agar.

3.17.5 Statistical Analysis

In the experimental animal study, statistical analysis was carried out using the Statistical Product and Service Solutions software (IBM SPSS statistics 20). Categorical data were compared by the χ^2 test, while unpaired differences in continuous data were compared by both the Mann-Whitney U test and the analysis of variance (ANOVA) test. All values were reported as Standard Error Mean (S.E.M ±) and a probability value of $p < 0.05$ was considered to be statistically significant.

Table 3.4 Experimental design of animal model of chronic lung infection.

Exeprimental Animals				Route of infection / day	Route of treatment / day	Dosage of treatment	
No	Group	Sex	Rats #			Volume (ml/kg)	Concentration (mg/kg)
1	Negative Control (Vehicle)	Male	6	-	Oral administration [b]	5 ml/kg	0.9% of NaCl (normal saline)
2	Negative Control (Vehicle)	Female	6	-	Oral administration [b]	5 ml/kg	0.9% of NaCl (normal saline)
3	Positive Control (infection)	Male	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	0.9% of NaCl (normal saline)
4	Positive Control (infection)	Female	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	0.9% of NaCl (normal saline)
5	Comparative Control (treatment)	Male	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	(25 gm/kg) of 2(5H)-Furanone
6	Comparative Control (treatment)	Female	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	(25 gm/kg) of 2(5H)-Furanone
7	Experimental Treatment (low dose)	Male	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	(25 gm/kg) of 139SI filtrate
8	Experimental Treatment (low dose)	Female	6	Intratracheal challenge [a]	Oral administration [b]	5 ml/kg	(25 gm/kg) of 139SI filtrate

[a] Intratracheal challenge was performed with 0.1 ml of *P. aeruginosa* 10^9 CFU/ml on day 1.
[b] Duration of oral administration was daily for 14 days.

3.18 Characterization of 139SI Active Compounds via HPLC

The soil bacterial filtrate that exhibited *in vitro* antibiofilm activity was further identified by High Performance Liquid Chromatography (HPLC) which was blindly performed by the technical staff at the laboratories of Cancer Research Initiatives Foundation (CARIF). The active filtrate was processed for sterile filtration then

converted into powder by lyophilisation by dissolving 60 mg of crude extract in 5 ml of water in volumetric flask. Solution was then filtered by using SRP-4 membrane 0.45µm. Stock solution (12,000 ppm) was kept in fridge 4°C before it was injected into HPLC column (Agilent Zorbax XDB-C18 ,4.6 x 250 mm, 5.0 µm) at 100 µl injection volume with a flow rate of 1.2 ml/min. The standard solvent system used was Acetonitrile and Water (Ammonium formate 0.005M) at a pH of 3.55. Furthermore, the spectrum range was 200-500 nm with UV absorption of 200, 230, 254 and 320 nm and a data acquisition time of 0-32 min yielding a total of 32 fractions (compounds) were tested separately for their antibiofilm activity *in vitro*.

3.19 Antibiofilm Activity of 139SI Active Compounds via MTP

To determine the antibiofilm activity of compounds obtained from the HPLC profiling of crude 139SI filtrate against biofilm-forming pathogens, MTP assay was performed as described in session 3.14.1 (Baldassarri *et al.*, 2006; Nithya *et al.*, 2010b). The selected concentration for each compound to be tested was the BIC (4500 µg/ml). Experiment was performed in triplicate and data was then averaged and standard deviation was calculated.

3.20 Identification of 139SI Active Compounds via LC-MS

Once the active fraction from HPLC was confirmed for its antibiofilm activity via MTP assay, further analysis was carried out to identify the chemical structure via Ultra Performance Liquid Chromatography-Diode Array Detection (UPLC–DAD) and Liquid Chromatography-Mass Spectrometry (LC-MS) which was blindly performed by the technical staff at the laboratories of CARIF. An Acquity UPLC system (Waters, Milford,

MA), equipped with a photo Diode Array Detection (DAD) detector was used for analysis and quantification under UPLC conditions.

Data were processed with Empower 2 software (Waters). The Ultra Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (UPLC–ESI-MS) peak identification was performed using the described UPLC system coupled with a LCQ DECA plus mass spectrometer equipped with an electrospray interface (Thermo-Finnigan Corp., San Jose., CA., USA). Instrument control and data acquisition were performed using Xcalibur 2.0 software. The quantification of UPLC–DAD was performed on a reversed-phase column Acquity UPLC BEH C-18 (2.1 × 50 mm) with 1.7 μm spherical porous particles. A linear gradient elution of A (Water + 0.1% Formic acid) and B (Acetonitrile + 0.1% Formic acid) was performed as follows: 0 min, 95% A, 5% B; 1 min, 85% A, 15% B; 2 min 45% A, 55% B; 5 min, 95% A,5% B; v/v. The flow rate was 0.5 ml/min. Column temperature was set at 30°C. Injected volume was 5 μl (Chen *et al.*, 2010).

The diode array UV–vis detector (DAD) was used for the detection and the wavelength for quantification was set at 277 nm. UPLC–ESI-MS analysis was operated in positive ESI modes. The electrospray needle voltage was 4 kV, and the capillary temperature was 250°C. Typical background source pressure was 1.2 × 10−5 Torr as read by an ion gauge. The drying gas was nitrogen. Following ion trapping, ions were mass analyzed and detected in the electron multiplier at 880 V by time-of-flight (TOF). The LCQ mass analyzer was scanned to 2000 m/z in positive mode to confirm peak identity by observation of the corresponding ionized molecule ([M+H]+). Collision gas for MS–MS experiment is He. Normalized collision energy was 32.5% (Chen *et al.*, 2010).

CHAPTER FOUR

RESULTS

4.1 Prevalence of Tonsillar Diseases

The prevalence of tonsillar diseases among our selected patients varied and based on the clinical examination obtained prior to surgery, the clinical cases were classified into two main groups, and these include infection group and non-infection (obstruction) group. Infection group comprised of recurrent tonsillitis (RT) and chronic tonsillitis (CT) cases whereas obstruction group comprised of obstructive sleep apnea (OSA) cases. Our results showed that RT was the predominant clinical case showing the highest number with 49 (70%) patients followed by CT with 9 (12.85%) patients and OSA with 12 (17.14%) patients.

Among the RT cases, there were 39 (55.71%) patients with Recurrent Tonsillitis, 4 (5.71%) patients with Recurrent Tonsillitis with Bilateral Middle Ear Effusion (MEE), 4 (5.71%) patients with Recurrent Tonsillitis with Snoring and 2 (2.85%) patients with Recurrent Adenotonsillitis. Whereas among the CT cases, there was 7 (10%) patients with Chronic Tonsillitis, 1 (1.42%) patient with Chronic Tonsillitis with Snoring and 1 (1.42%) with patient Chronic Adenotonsillitis. Among the OSA cases, there were 3 (4.28%) patients with Obstructive Sleep apnea, 3 (4.28%) patients with OSA Secondary to Tonsillar Hypertrophy, 3 (4.28%) patients with OSA Secondary to Recurrent Tonsillitis, 1 (1.42%) patient with OSA Secondary to Adenoid Hyperplasia, 1 (1.42%) patient with OSA with Primary Snoring (PS) and 1 (1.42%) patient with OSA Secondary to Recurrent Acute Tonsillitis.

The prevalence of RT cases among the pediatric patients was 22 (31.42%) cases while in adult patients was 29 (41.42%) cases whereas the prevalence of CT cases

among pediatrics was 4 (5.71%) cases while in adults it was 5 (7.14%) cases. However, the prevalence of OSA cases among pediatrics was 9 (12.85%) cases while in adults was 3 (4.28%) cases. In the RT cases, it was noticed that the highest number of age group was 1.0-10 years old with 18 (25.71%) patients followed by 11.2-20 years old with 16 (22.85%) patients and 21-30 years old with 14 (20%) whereas the age group 31-40 years old was among the lowest with 3 (4.28%) patients along with the age group 41-50 years old with only 1 (1.42%) patient.

In the CT cases, it was noticed that the highest number of age group was 11.0-20 with 5 (7.14%) patients followed by the age group 1.0-10 years old with 3 (4.28%) patients and only 1 (1.42%) patient was within the age 21-30 years old. Whereas no patients were in the 31-40, 41-50 and 51-60 age groups. In the OSA cases, it was noticed that the predominant age group was 1.0-10 with 7 (10%) patients followed by the age group 11.0-20 years old with 2 (2.85%) patients whereas no patients fell in the rest of age groups.

The distribution of patients among clinical cases based on their age groups is illustrated in (Figure 4.1). In terms of frequency and type of operative procedures performed on 70 clinical cases, 44 (62.85%) patients underwent tonsillectomy alone while 26 (37.14%) underwent Tonsillectomy & Adeonoidectomy (T&A). The detailed list of all clinical cases, type and date of operation can be found in (Appendix 8).

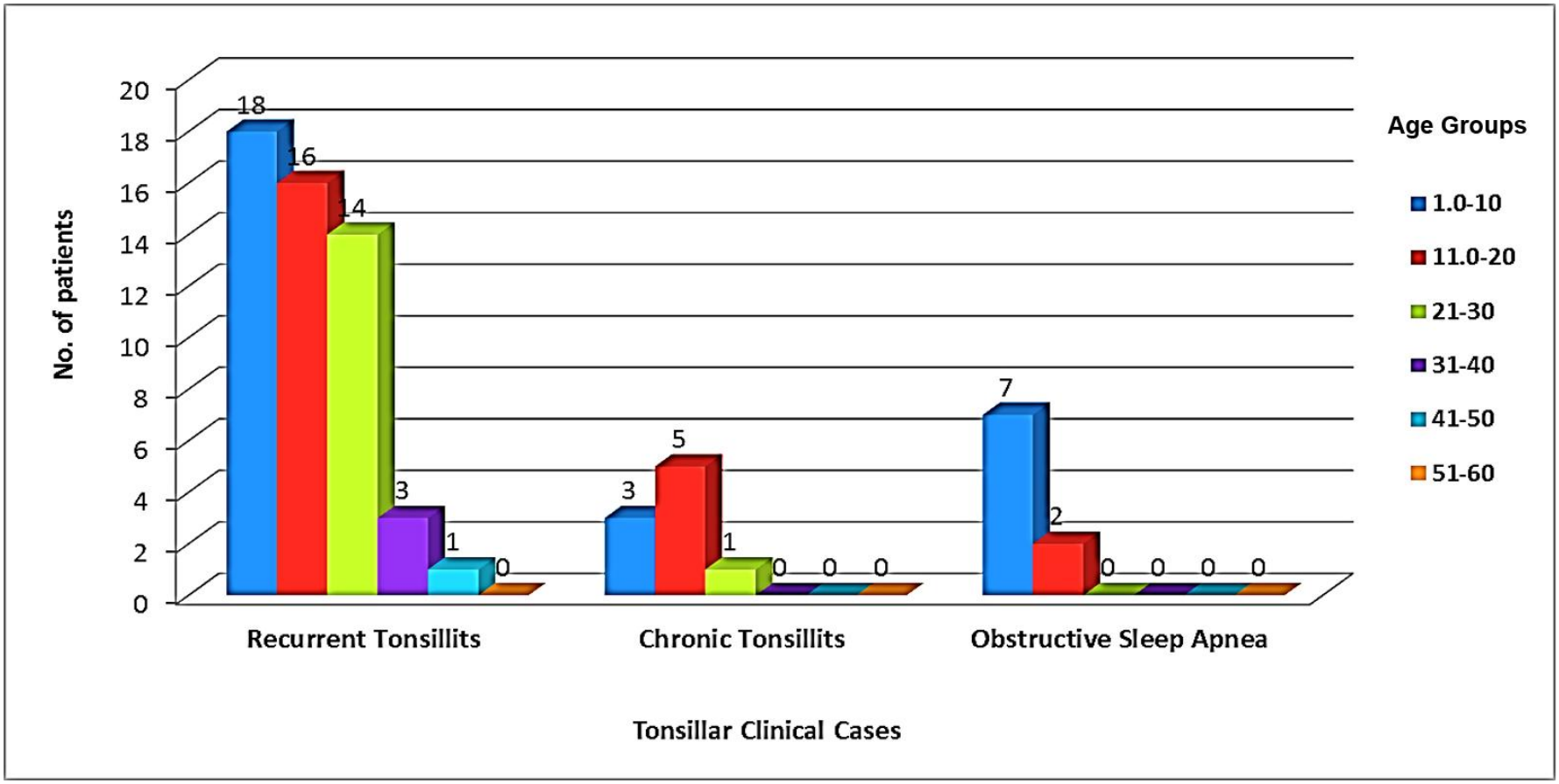

Figure 4.1 Distribution of patients among clinical cases based on age groups.

4.2 Histopathology of Tonsillar Specimens

Based on the gross pathology of collected tonsillar biopsies, our results showed that all patients had enlarged tonsils with various grading and an association with adenoids hypertrophy was detected in 26 (37.14%) patients. Patients presenting enlarged (hypertrophied) tonsils were classified into 4 groups according to size: grade I (1+) with 6 (8.57%) patients; grade II (2+) with 20 (28.57%) patients; grade III (3+) with 39 (55.71%) patients and grade IV (4+) with 5 (7.15%) patients.

Based on the anatomic pathology report performed by the Department of Pathology, Faculty of Medicine, University of Malaya, the macroscopy (gross biopsy) evaluation of all the 140 tonsils described the specimens as nodular to tubular irregular brownish & soft surface tissue with an average measuring size of 2cm x 2cm x 1cm. However, the microscopy (histology section) evaluation described the tonsillar lymphoid tissue as being covered with benign stratified squamous epithelium with the stroma consisting of variably sized reactive lymphoid follicles where no cases of malignancies being found, a rate of crypt keratination was also reported.

The overall pathological interpretation for the excised tonsils was stated as reactive (benign) lymphoid hyperplasia. The histopathology evaluation showed evidence of infection with *Actinomyces* sp. among 11 (15.71%) of our selected patients. These infections caused and an inflammatory lesion of the tonsillar crypts and led to tonsillar hypertrophy. However, it was noticed that there was no description for other microbial infections such biofilms. The gross pathology of excised palatine tonsils representing the 4 tonsillar grading is shown in (Figure 4.2).

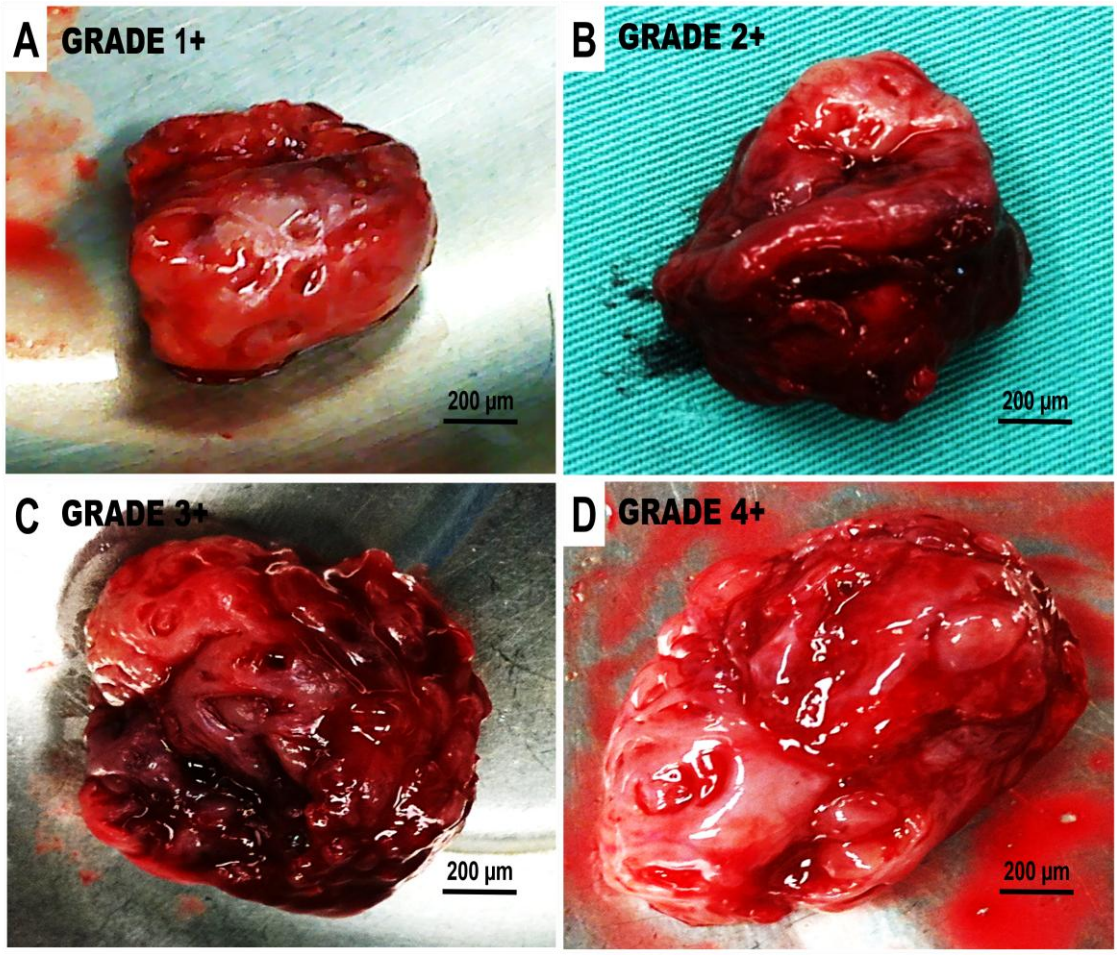

Figure 4.2 Gross pathology of tonsils showing four tonsillar hypertrophy grading. **A,** grade I (+1) patient with recurrent tonsillitis. **B,** grade II (+2) patient with chronic tonsillitis. **C,** grade III (+3) patient with obstructive sleep apnea. **D,** grade IV (+4) patient with OSA secondary to tonsillar hypertrophy.

4.3 Bacterial Isolates from Tonsillar Specimens

Based on our results, a total number of 464 bacterial isolates (302 Gram-positive and 162 Gram-negative) were recovered from all the tonsillar specimens. The most common isolate was *Staphylococcus aureus* (39.65%) followed by *Haemophilus influenzae* (18.53%) then *Streptococcus agalactiae* (12.06%). Differences in distribution between the surface (swab) and core (biopsy) were apparent at the species or group level. The number of recovered bacterial isolates from surface sites was 239 (51.50%) in comparison with 225 (48.49%) isolates from core sites indicating no

significance in the recovery when considering the total number of isolates per site. All isolates usually were found to be present similarly at both sites of the tonsils except for *Haemophilus parainfluenzae* where it was most frequently isolated from core specimens 10 (2.15%) more than the surface 21 (4.52%). Distribution of bacterial isolates among tonsillar specimens is shown (Table 4.1) and (Figure 4.3).

Table 4.1 Distribution of bacterial isolates among tonsillar specimens.

Gram-Positive Isolates	Tonsillar Biopsy (core) No. (%)	Tonsillar Swab (surface) No. (%)	Total No. (%)
Staphylococcus aureus (SA)	85 (18.31%)	99 (21.33%)	184 (39.65%)
Methicillin Resistant *S. aureus* (MRSA)	0	1 (0.21%)	1 (0.21%)
Streptococcus agalactiae (GBS) [1]	36 (7.75%)	20 (4.31%)	56 (12.06%)
Group F Streptococci (GFS) [3]	5 (1.07%)	6 (1.29%)	11 (2.37%)
Group C Streptococci (GCS) [1]	4 (0.86%)	4 (0.86%)	8 (1.72%)
Group G Streptococci (GGS) [1]	11(2.37%)	14 (3.01%)	25 (5.38%)
Streptococcus pyogenes (GABHS) [1]	6 (1.29%)	8 (1.72%)	14 (3.01%)
Streptococcus pneumoniae [2] (SPn)	1 (0.21%)	2 (0.43%)	3 (0.64%)
SUBTOTAL	148 (31.89%)	154 (33.18%)	302 (65.08%)

Gram-Negative Isolates	Tonsillar Biopsy (core) No. (%)	Tonsillar Swab (surface) No. (%)	Total No. (%)
Acinetobacter baumannii (AB)	1 (0.21%)	0	1 (0.21%)
Citrobacter sp. [4]	2 (0.43%)	2 (0.43%)	4 (0.86%)
Enterobacter cloacae [4]	0	1 (0.21%)	1 (0.21%)
Haemophilus influenzae (HI)	44 (9.48%)	42 (9.05%)	86 (18.53%)
Haemophilus parainfluenzae (HP)	10 (2.15%)	21 (4.52%)	31 (6.68%)
Klebsiella pneumoniae [4] (KP)	15 (3.23%)	15 (3.23%)	30 (6.46%)
Pseudomonas aeruginosa [4] (PA)	5 (1.07%)	4 (0.86%)	9 (1.93%)
SUBTOTAL	77 (16.59%)	85 (18.31%)	162 (34.91%)
TOTAL	225 (48.49%)	239 (51.50%)	464 (100%)

[1] *Pyogenic streptococci group (Lancefield groups) / β- hemolytic Streptococci,*
[2] *Pneumococci group / α- hemolytic Streptococci,*
[3] *Viridans Group Streptococci (VGS) / Group F Streptococci (GFS),*
[4] *Enterobacteriacea*

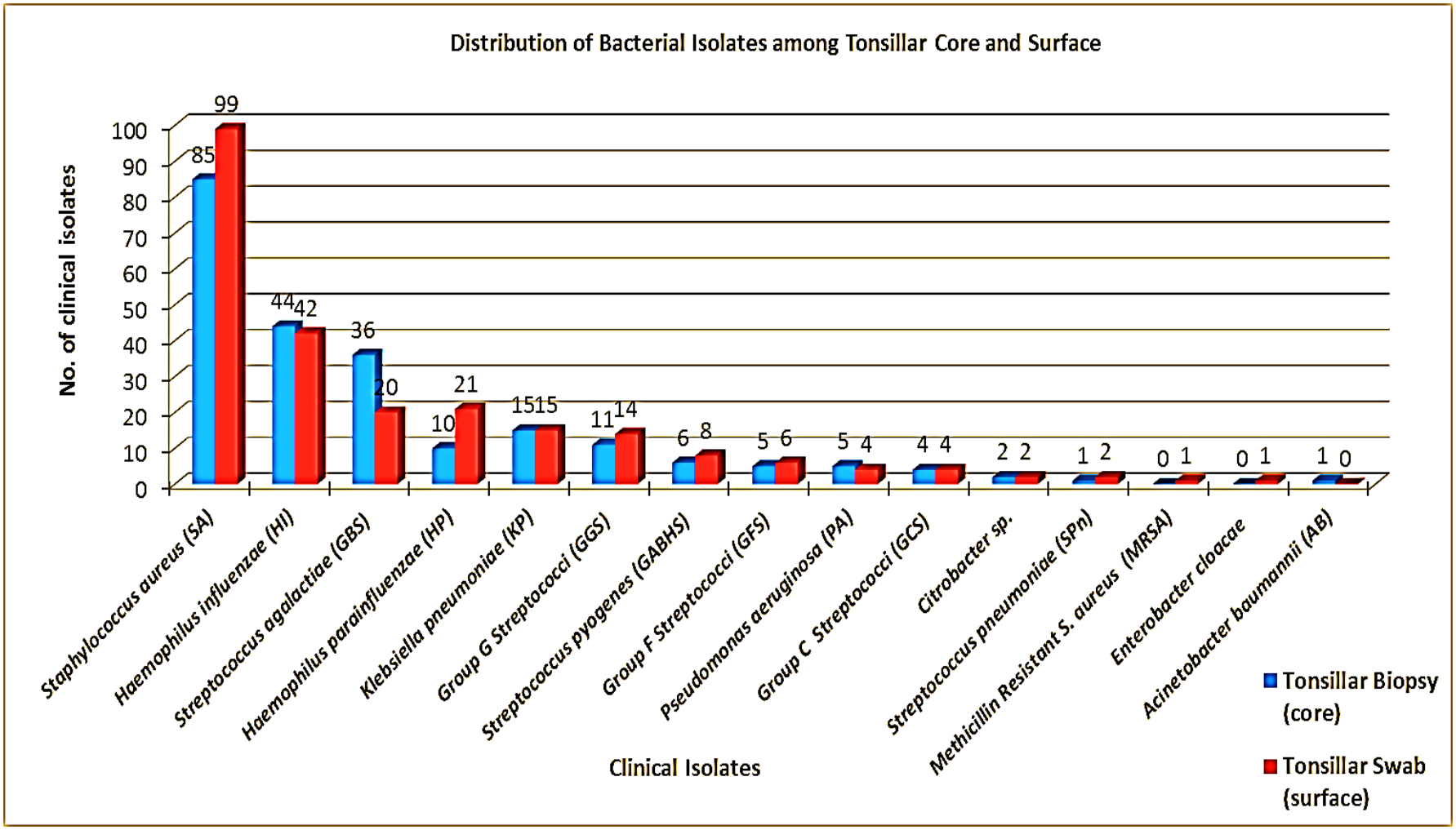

Figure 4.3 Distribution of bacterial isolates among tonsillar specimens.

The weight of excised tonsils varied from 2.2 to 8.1 grams. However, there was no correlation between tonsillar weight (size) and the number or type of isolated microorganisms. However, there was a difference in the recovery rate of microorganisms among RT, CT and OSA cases where RT showed an average of 10.85 isolates/gram tonsil while CT showed and average of 3.75 isolates/gram tonsil and OSA showed an average of 3 isolates/gram tonsil.

The low recovered isolates varied in their distribution among clinical cases, for example *Haemophilus parainfluenzae* (HP) was present in 25 (5.38 %) RT cases followed by 3 (0.64%) CT cases then 2 (0.43%) OSA cases. Group G Streptococci (GGS) were present only in 18 (3.87 %) RT cases while absent in CT and OSA. Group G Streptococci (GCS) were present equally 4 (0.86 %) in both RT and OSA while absent in CT. *Streptococcus pneumoniae* (SPn) was present equally 1 (0.21 %) in CT and OSA while absent in RT. *Pseudomonas aeruginosa* (PA) was present in 5 (1.07 %) RT cases and 4 (0.86 %) OSA cases while absent in CT. *Citrobacter s*p. was only present in 4 (0.86 %) RT cases while absent in CT and OSA. *Enterobacter cloacaea*, *Acinetobacter baumannii* and Methicillin Resistant *S. aureus* as there was only one isolate from each was only recovered from RT respectively. There was no assessment of anaerobes, fungi and viruses to support their role in our study. The distribution of Gram-positive and Gram-negative bacterial isolates among both tonsillar infection and obstruction groups is shown (Figure 4.4) and (Figure 4.5).

A polymicrobial flora of mixed organisms were recovered from different clinical groups except for Group A Beta-Haemolytic Streptococci (GABHS) where all the 14 isolates has been recovered only from the group of Recurrent Tonsillitis cases among pediatric and young adults patients. The recovery rate of Group A Beta-Haemolytic Streptococci (GABHS) did not vary with patients' age and the isolation of non-GABHS was two times higher in adults than in children.

The mean age in Streptococcal Tonsillitis (ST) group was 10 years old while in the Non-Streptococcal Tonsillitis (NST) group it was 13.34 years. The prevalence of ST group was significantly less than NST group which emphasizes the role of group B, C, G and F in the clinical presentation of pahryngotonsillitis (PT) infections.

An interesting finding includes the recovery of a group of pathogens called ESKAPE which includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. It was emphasized that these pathogens pause a threat causing the majority of US hospital infections and effectively "escape" the effects of antibacterial drugs (Boucher *et al.*, 2009). 225 (48.46%) ESKAPE pathogens were recovered from both tonsillar swab and specimens with 184 isolates (39.65%) of *Staphylococcus aureus*, 30 (6.46%) of *Klebsiella pneumoniae*, 9 (1.93%) of *Pseudomonas aeruginosa* and 1 (0.21%) isolate of *Acinetobacter baumannii* and *Enterobacter cloacae*, while there was no recovery for *Enterococcus faecium* isolates.

The only significant bacterial pathogen in acute pahryngotonsillitis is *Streptococcus pyogenes* or GABHS (Hoffmann, 1987). However, only 14 (3.01 %) isolates were recovered among patients with only recurrent tonsillitis (RT), while there were no isolates recovered from both CT and OSA. Detailed table for the distribution and mean of recovered bacterial isolates from tonsillar specimens is described (Appendix 9).

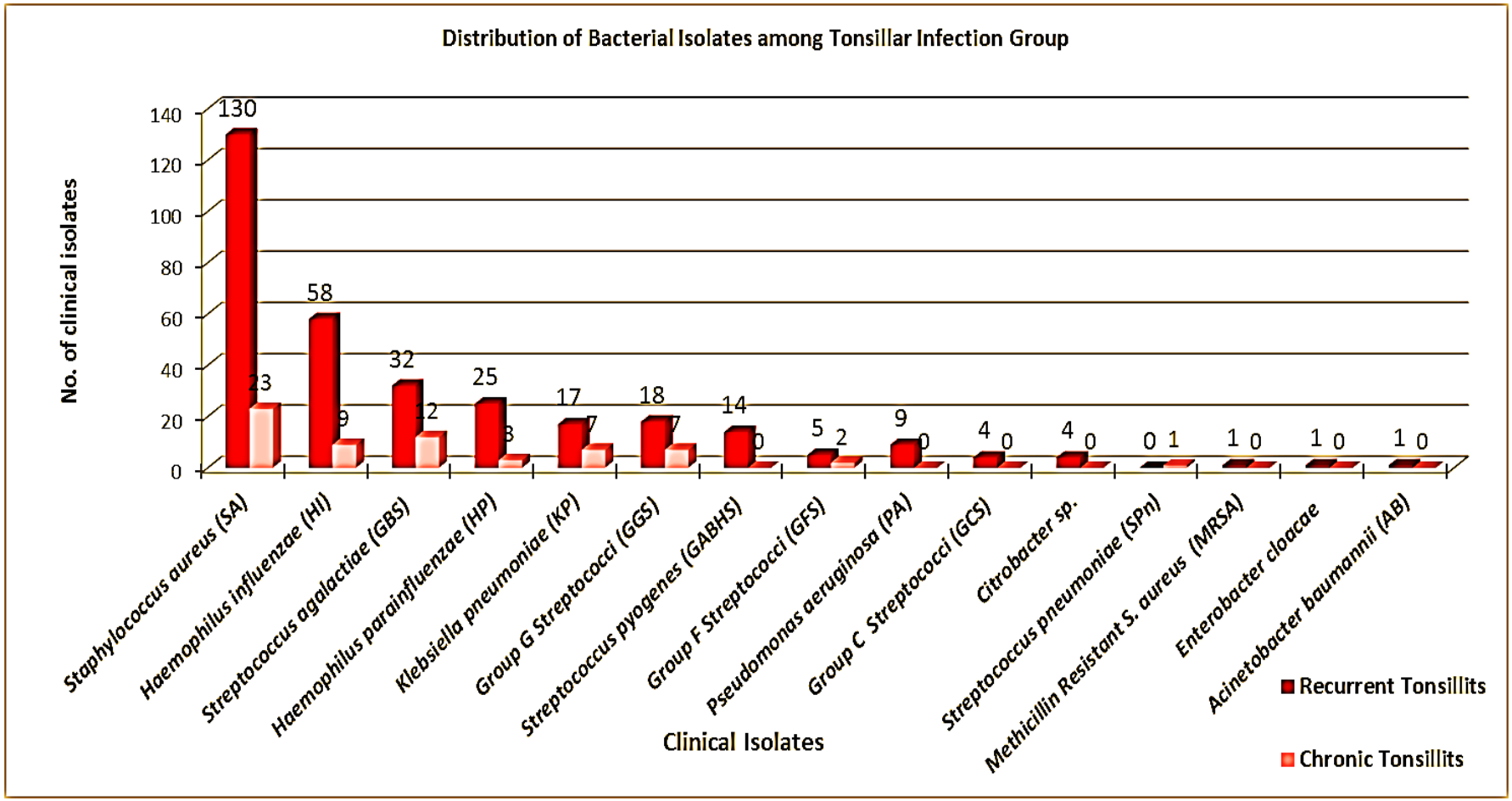

Figure 4.4 Distribution of bacterial isolates among patients with chronic and recurrent tonsillitis.

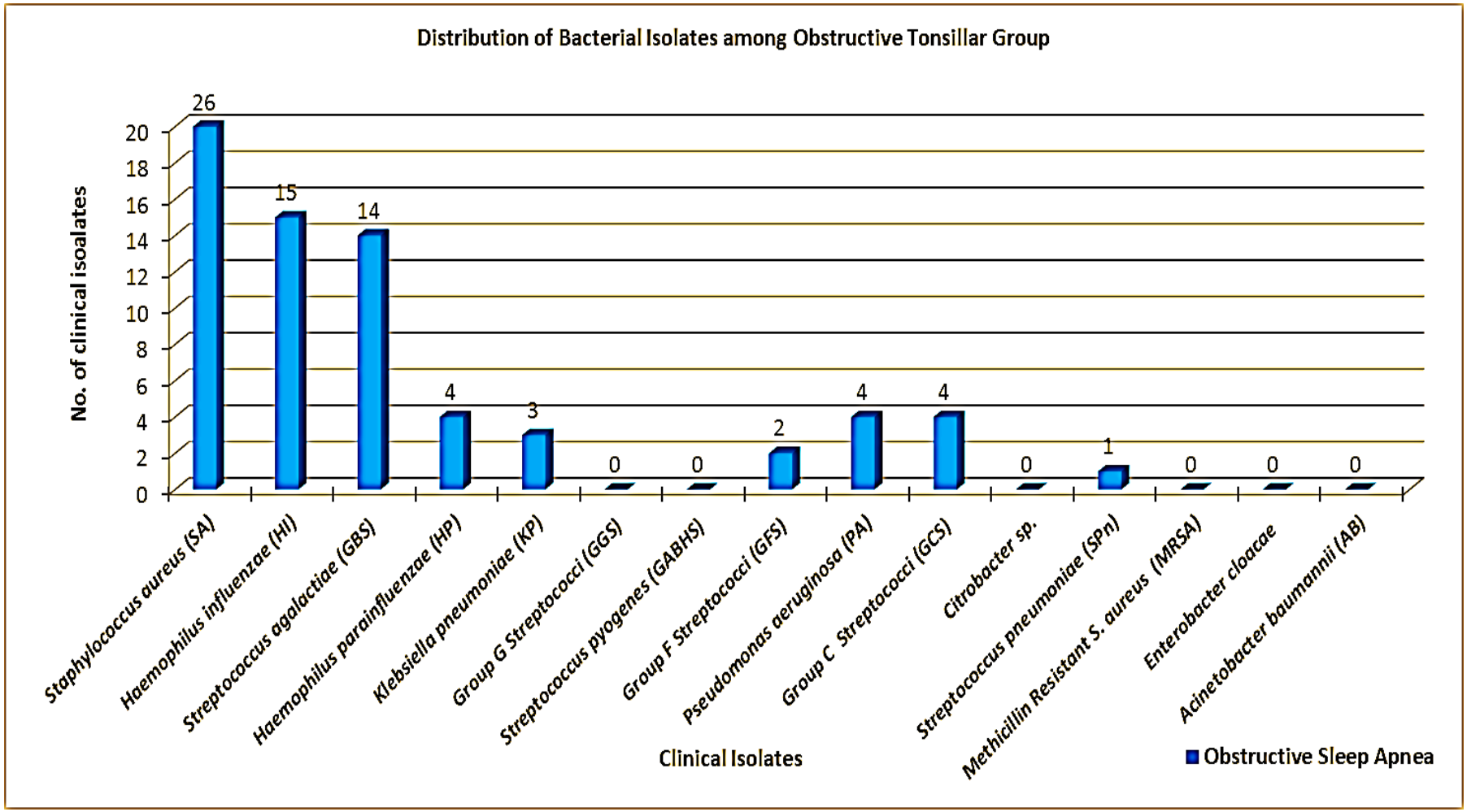

Figure 4.5 Distribution of bacterial isolates among patients with obstruction sleep apnea.

4.4 Antimicrobial Susceptibility of Bacterial Isolates

Among the 184 isolates of *Staphylococcus aureus*, 169 (91.48%) were susceptible to all the selected antibiotics, 20 (10.87%) were resistant to fusidic acid only, whereas 1 (0.5%) isolate (MRSA) was resistant to both methicillin and fusidic acid (Alasil *et al.*, 2011) (Appendix 3A). There was no significance in the difference of susceptibility between swab and biopsy specimens among adults and children and among male and female patients.

The antibiotic co-trimoxazole (SXT) (trimethoprim-sulfamethoxazole) has shown the highest rate of resistance against various bacterial isolates including GABHS showing 11 (2.37%) resistant isolates and 3 (0.64%) susceptible, Group B Streptococcus showing 55 (11.85%) resistant isolates and 1 (0.21%) susceptible, Group G Streptococcus showing 14 (3.01%) resistant isolates and 11 (2.37%) susceptible, *Streptococcus pneumoniae* showing 3 (0.64%) resistant isolates, *Haemophilus influenzae* showing 27 (5.81%) resistant isolates and 59 (12.71%) susceptible and *Haemophilus pararinfluenzae* showing 10 (2.15%) resistant isolates and 21 (4.52%) susceptible.

The number of *Haemophilus influenzae* (HI) isolates that were β-Lactamase Negative Ampicillin-Resistant (BLNAR) was 12 (2.58%) isolates were. A resistance to the penicillins class was detected including *Streptococcus pneumoniae* with 3 (0.64%) isolates resistant to penicillin, *Haemophilus influenzae* with 12 (2.58%) isolates resistant to ampicillin, *Klebsiella pneumoniae* with 30 (6.46%) isolates resistant to ampicillin, *Pseudomonas aeruginosa* with 7 (1.50%) isolates resistant to ampicillin, *Acinetobacter baumannii* with 1 (0.21%) isolate resistant to ampicillin, *Citrobacter* sp. with 4 (0.86%) isolates resistant to ampicillin and *Enterobacter cloacae* with 1 (0.21%) isolate resistant to ampicillin.

There was no significance in the number of Beta-Lactamase-Producing Bacteria (BLPB) with a total number of 5 isolates including 1 isolate of *Acinetobacter baumannii* against the antibiotic cefotaxime (CTX), 3 isolates of *Haemophilus influenzae* and 1 isolate of *Haemophilus parainfluenzae*. A low rate in the number of isolates among infected tonsils that were multidrug resistant (MDR) was 10 (2.15%), while the number of isolates among non-infected tonsils that were multidrug resistant was 7 (1.50%) with a total of 17 (3.66%). The MDR isolates were represented by 7 isolates of Multidrug Resistant Gram-Negative Bacilli (MDR-GNB) *Pseudomonas aeruginosa*, 3 isolates of MDR *Streptococcus pneumoniae*, 1 isolate of multidrug resistant (MDR) *Enterobacter cloacae* whereas no MDR isolates of *Klebsiella pneumoniae* Carbapenemase (KPC) were identified. Interestingly, there was an increased number of *H. influenzae* isolates in association with GABHS which may be due to a synergistic relationship between these organisms. A summary of antimicrobial susceptibility among all Gram-Positive and Gram-Negative isolates against selected β-lactam and non-β-lactam agents is shown (Table 4.2) and illustrated (Figure 4.6) and (Figure 4.7). Detailed tables for the antimicrobial susceptibility results and antibiogram patterns of tonsillar isolates is described (Appendixes 12A, 12B, 12C, 12D, 12E, 12F, 12G, 12H, 12I, 12J, 12K, 12L, 12M, 12N, 12O).

Table 4.2 Summary table of antimicrobial susceptibility percentage among tonsillar isolates.

Clinical isolates	Antimicrobial agents																						
	β-Lactams													Non-β-Lactams									
	[1]Ampicillin (AM)	[2]Amoxicillin-Clavulanic acid (AMC)	[2]Ampicillin-Sulbactam (SAM)	[3]Cefoperazone (CFP)	[3]Cefotaxime (CTX)	[3]Ceftazidime (CAZ)	[3]Ceftriaxone (CTR)	[3]Cefuroxime (CXM)	[4]Cephalexin (LEX)	[5]Imipenem (IPM)	[1]Methicillin (ME)	[1]Penicillin (PEN)	[2]Piperacillin-tazobactam (TZP)	[6]Amikacin (AN)	[12]Azithromycin (AZM)	[11]Clindamycin (CM)	[8]Ciprofloxacin (CIP)	[9]Co-trimozaxole (SXT)	[12]Erythromycin (EM)	[13]Fusidic Acid (FA)	[6]Gentamicin (GM)	[7]Rifampin (RA)	[10]Vancomycin (VA)
Gram-Positive																							
MRSA [a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	100	100	–	100	100	100	–
S. aureus	–	–	–	–	–	–	–	–	–	–	100	–	–	–	–	100	–	100	100	89.13	100	100	–
S. agalactiae (GBS)	100	–	–	–	–	–	–	100	100	100	–	100	–	–	–	100	–	1.78	98.21	–	–	–	100
Group F Streptococci	100	–	–	–	–	–	–	100	100	100	–	100	–	–	–	100	–	100	100	–	–	–	100
Group C Streptococci	100	–	–	–	–	–	–	100	100	100	–	100	–	–	–	100	–	100	100	–	–	–	100
Group G Streptococci	100	–	–	–	–	–	–	100	100	100	–	100	–	–	–	100	–	44	100	–	–	–	100
S. pyogenes (GABHS)	100	–	–	–	–	–	–	100	100	100	–	100	–	–	–	100	–	21.43	100	–	–	–	100
S. pneumoniae	–	–	–	–	–	–	–	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100
Gram-Negative																							
A. baumannii	–	–	100	100	100	100	–	100	–	100	–	–	100	100	–	–	100	100	–	–	100	–	–
Citrobacter sp.	100	100	100	100	100	100	100	100	–	100	–	–	100	100	–	–	100	100	–	–	100	–	–
E. cloacae	–	–	100	100	100	100	100	100	–	100	–	–	100	100	–	–	100	100	–	–	100	–	–
H. influenzae	86.04	98.83	–	–	–	–	100	100	–	–	–	–	–	–	100	–	–	68.60	–	–	–	–	–
H. parainfluenzae	93.55	100	–	–	–	–	100	100	–	–	–	–	–	–	100	–	–	67.75	–	–	–	–	–
K. pneumoniae	–	100	100	100	100	100	100	100	–	100	–	–	100	100	–	–	100	100	–	–	100	–	–
P. aeruginosa	22.22	22.22	22.22	100	100	100	100	100	–	100	–	–	100	22.22	–	–	100	100	–	–	22.22	–	–
TOTAL MEAN (%)	77	84	84	100	100	100	100	100	100	100	100	100	100	84	100	100	100	73	99	94	88	100	100

[1]Penicillins class, [2]β-lactamase inhibitor combinations class, [3]Cephems (parenteral) class, [4]Cephems (oral) class, [5]Penems class, [6]Aminoglycosides class, [7]Ansamycins class, [8]Quinolones class, [9]Folate pathway inhibitors class, [10]Glycopeptides class, [11]Lincosamides class, [12]Macrolides class, [13]Fusidane class.

(–) Drugs that has not been tested or indicated.

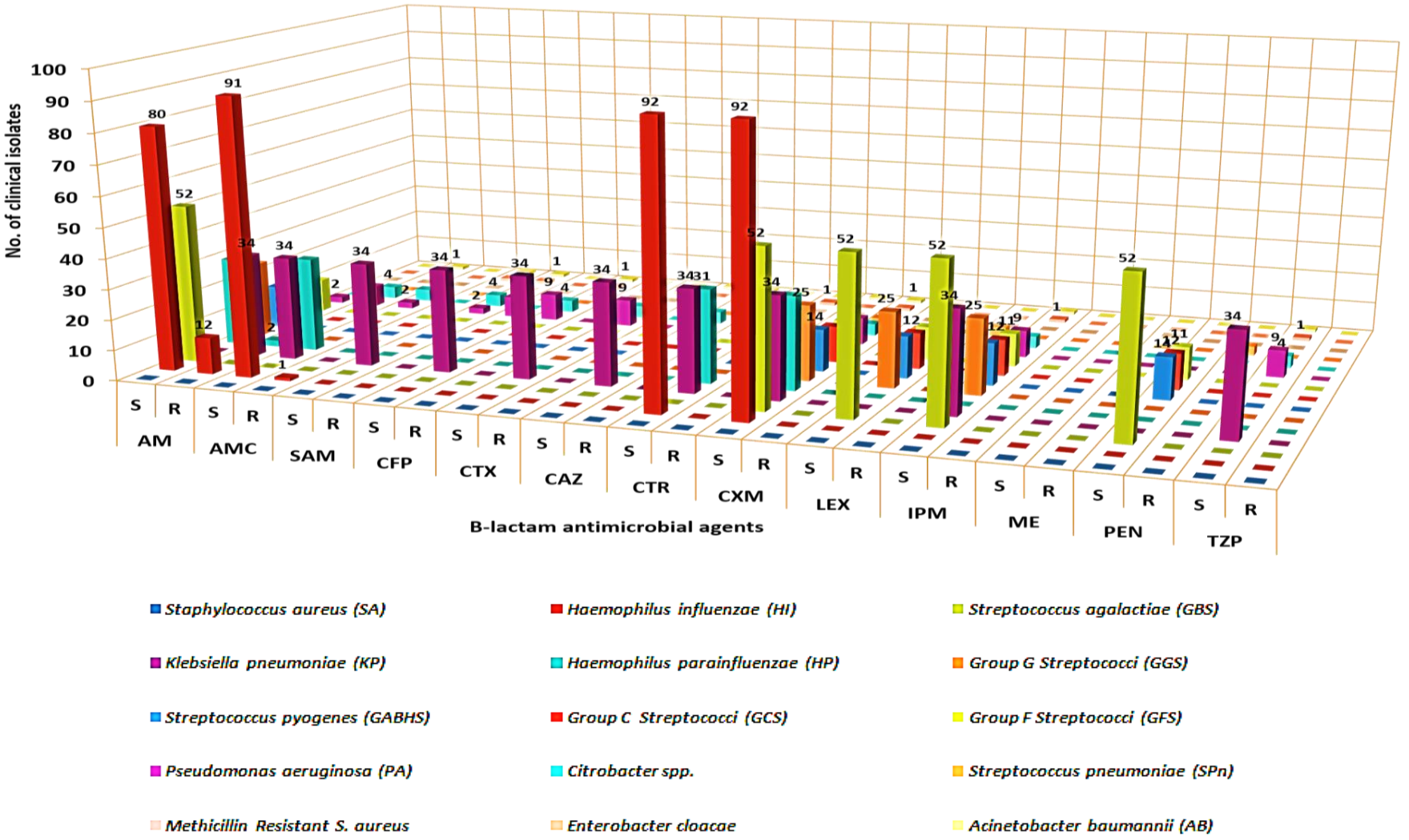

Figure 4.6 Antimicrobial susceptibility of tonsillar isolates against selected β-lactam agents.

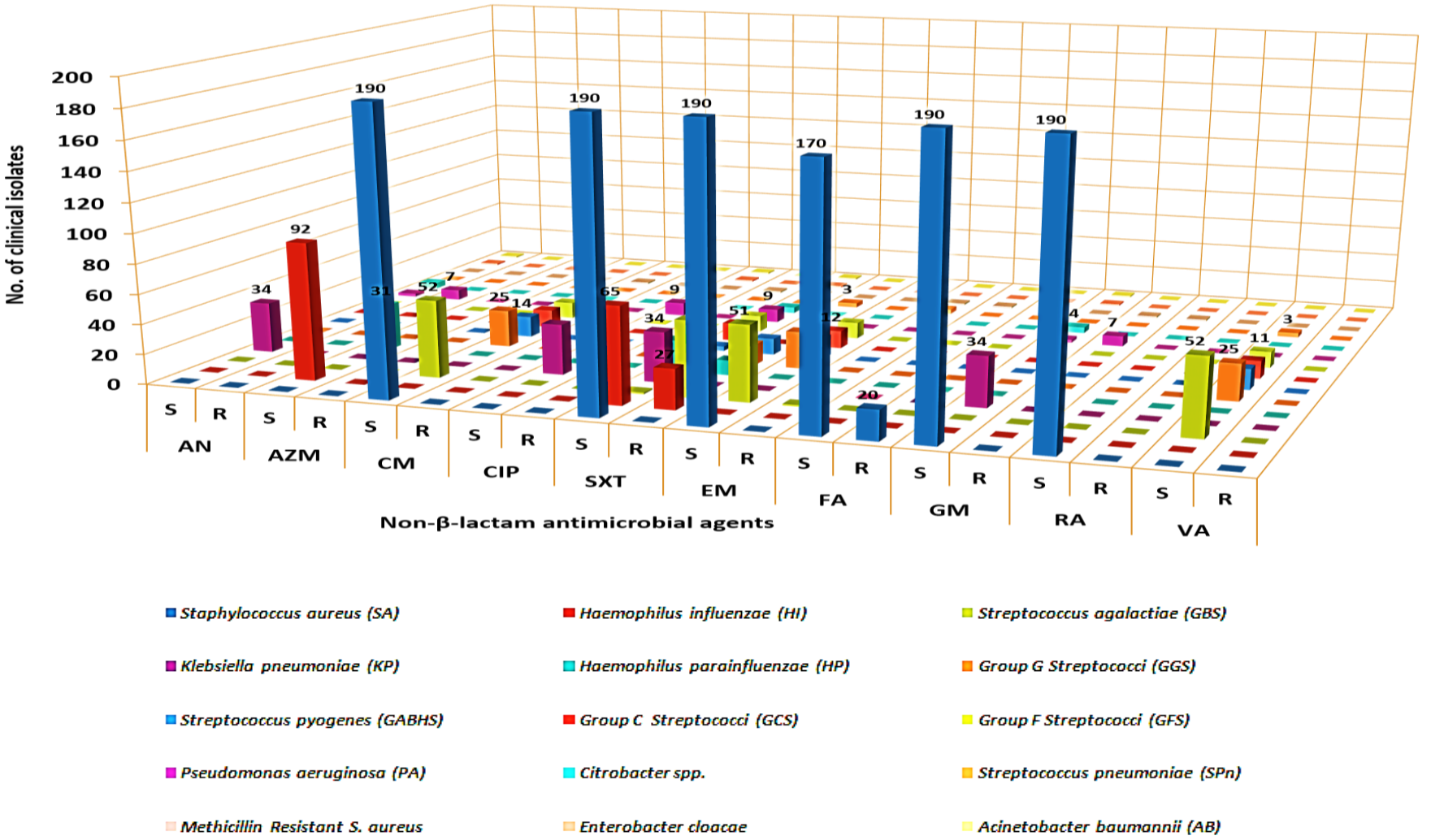

Figure 4.7 Antimicrobial susceptibility of tonsillar isolates against selected non-β-lactam agents.

4.5 Microscopic Detection of Biofilm Presence

4.5.1 Scanning Electron Microscopy

Our results showed the presence of attached bacteria in the form of biofilms on the surface of tonsils in 42 (60%) of selected patients. Among the tonsillar infection group, the presence of bacterial biofilm was detected in 30 out of 49 patients with recurrent tonsillitis and 5 out of 9 patients with chronic tonsillitis. However, among obstructive (non-infected) group, biofilms were present in 7 out of 12 patients with obstructive sleep apnea. The microscopic analysis of various tonsillar specimens via SEM showed the network-like biofilm glycocalyx embedding the bacterial microcolonies (Figure 4.8 A). Attached bacteria were present on the surface of the specimen and could be clearly distinguished from smaller materials or irregularities of the tonsillar epithelium nearby. The low magnification images showed the tonsillar mucosa comprising of small debris covered with a network-like glycocalyx and blood cells (Figure 4.8 B). The number of attached bacteria was variable among specimens. The cells were not evenly distributed over the entire surface of the specimen but rather were clustered and formed microcolonies. These microcolonies were mostly located in small depressions between epithelial cells. Using higher magnification, the location of these microcolonies was confirmed to be at the junction of the epithelial cells in small crypts. Bacterial cells were visualized as being connected by an extracellular material which could represent the glycocalyx matrix and they were seemed to be organized in a scaffolding network located in small crypts. The adherent biofilms had a varying number of attached bacteria, ranging from a few cells to a mass. However, some of the dividing bacterial cells were observed within microcolonies (Figure 4.8 B). The appearance of the biofilm was inconsistent among our specimens. Some areas were covered completely with attached bacteria whereas others had few attached microbes, as evidenced by the presence of areas of normal mucosa.

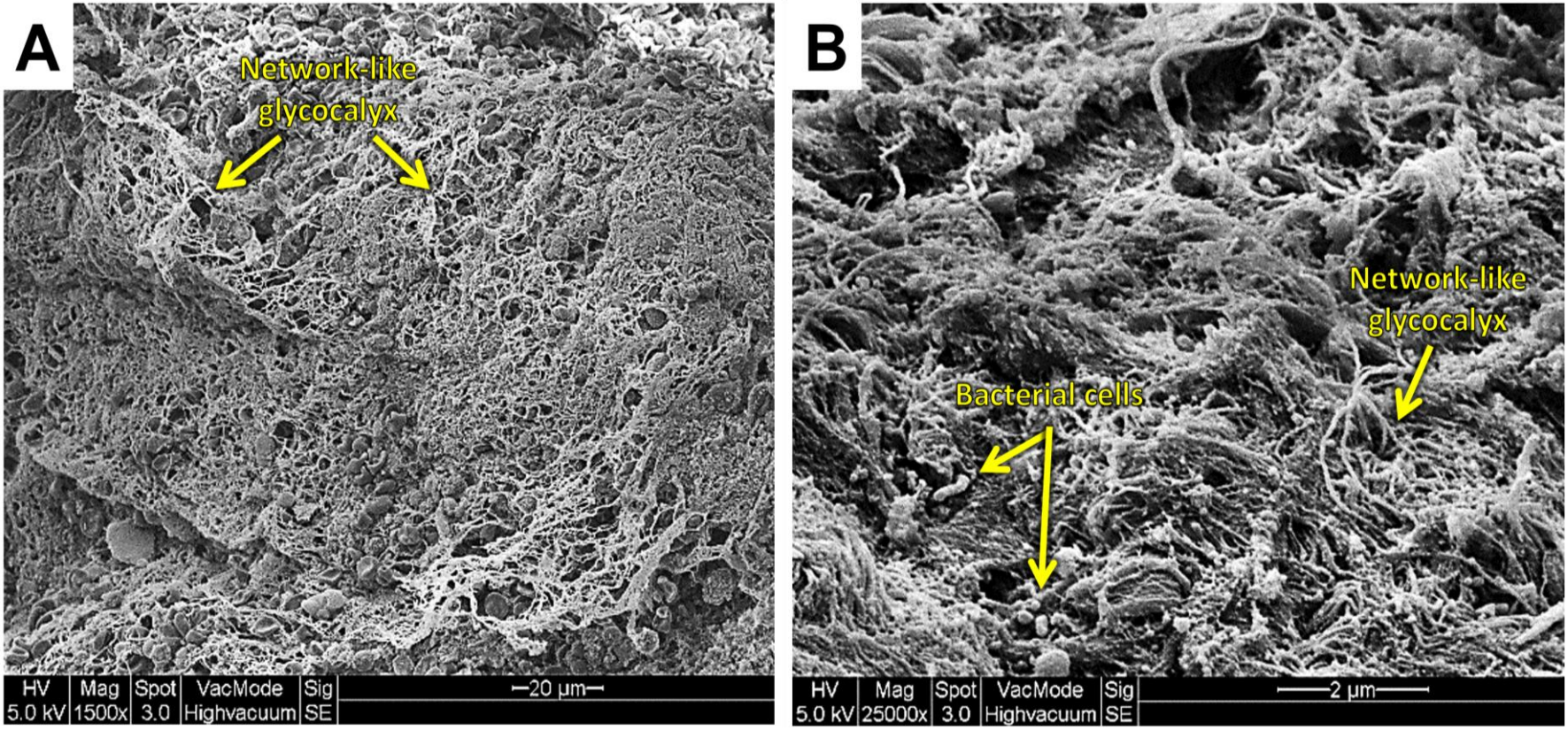

Figure 4.8 Microscopic evidence of bacterial biofilms on the tonsillar surface via SEM. **A,** Overall image of biofilm from a patient with recurrent tonsillitis showing the layers of network-like glycocalyx (Low magnification 1500x). **B,** Representative image of biofilm from a patient with chronic tonsillitis showing bacterial cells attached to the surface of tonsillar cells and embedded in a network-like glycocalyx (High magnification 25000x). Arrows indicate the biofilm structures.

4.5.2 Confocal Laser Scanning Microscopy

The results of viewing biofilms using CLSM were consistent with the results shown using SEM. We have demonstrated the presence of 3-dimensional structures of biofilms in the tonsils of 42 (60%) patients for which 30 out of 49 patients were diagnosed with recurrent tonsillitis, 5 out of 9 patients were diagnosed with chronic tonsillitis. And 7 out of 12 patients with diagnosed with obstructive sleep apnea. To visualize bacterial cells and their surrounding glycocalyx matrix, which indicates the presence of a biofilm, double staining was performed using Propidium iodide and Concanavalin A (Con A). Bacterial cells and nuclei of tonsillar cells were stained red, whereas binding of Con A resulted in a fluorescent green staining indicated the presence of the biofilm's glycocalyx. Inter-connected bacteria were encased in a scaffolding network composed of extracellular matrix, suggesting a 3-D structure of biofilm (Figure 4.9 A). Higher magnification on CLSM shows the biofilm with its glycocalyx in green embedding the bacterial cells in red and covering the space between tonsillar nuclei in red (Figure 4.9 B). Most of the bacteria were cocci shaped arranged in clusters and it noticed that ConA stained the biofilm's matrix exclusively. One of the important features of using CLSM in visualizing biofilms is that it allows for a quantitative co-localization analysis to examine antigens of interest in immunofluorescence images; in our case is the biofilm's glycocalyx. The histometric measurement of the biofilm's fluorescent-labeled structures showed green glycocalyx tagged with Con A and red bacterial cells tagged with Propidium iodide (Figure 4.10 A and B). The co-localized areas of detected biofilms were measured where single tagged pixels of the two channels are located in quadrant 1 and 2 respectively while pixels having intensity above the background (co-localized pixels) are located in quadrant 3 (Figure 4.11 A). The magnified 2.5-D image of biofilms were also measured (Figure 4.11 B).

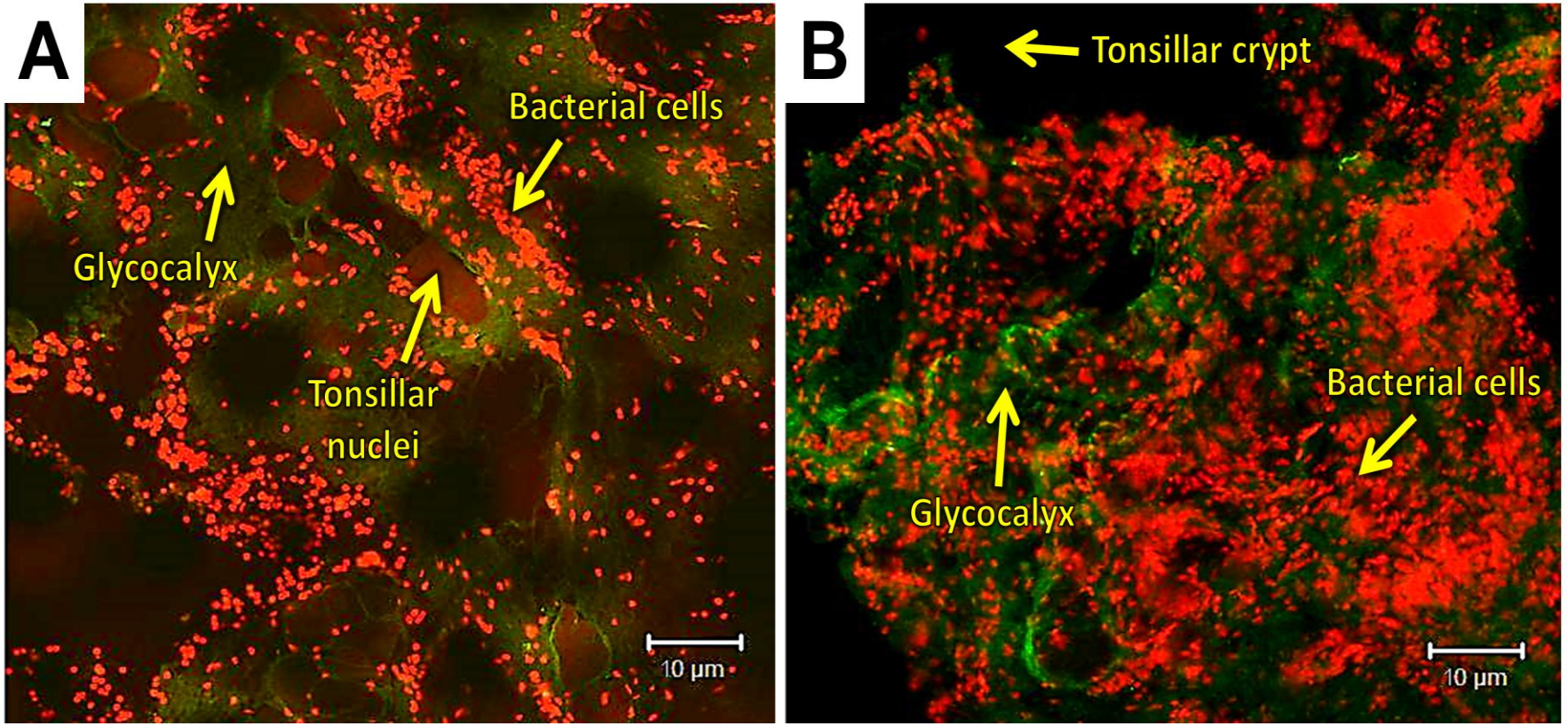

Figure 4.9 Microscopic evidence of bacterial biofilms within the tonsillar crypts via CLSM. **A,** Representative image of biofilm from a patient with obstructive sleep apnea showing bacterial cells (red) embedded with glycocalyx (green) surrounding the tonsillar nuclei (red). **B,** Three-dimensional image of a biofilm showing bacterial aggregates (cells) embedded in a glycocalyx matrix (100x). Arrows indicate the biofilm structures and tissue sections were stained with Propidium iodide & Concanavalin A.

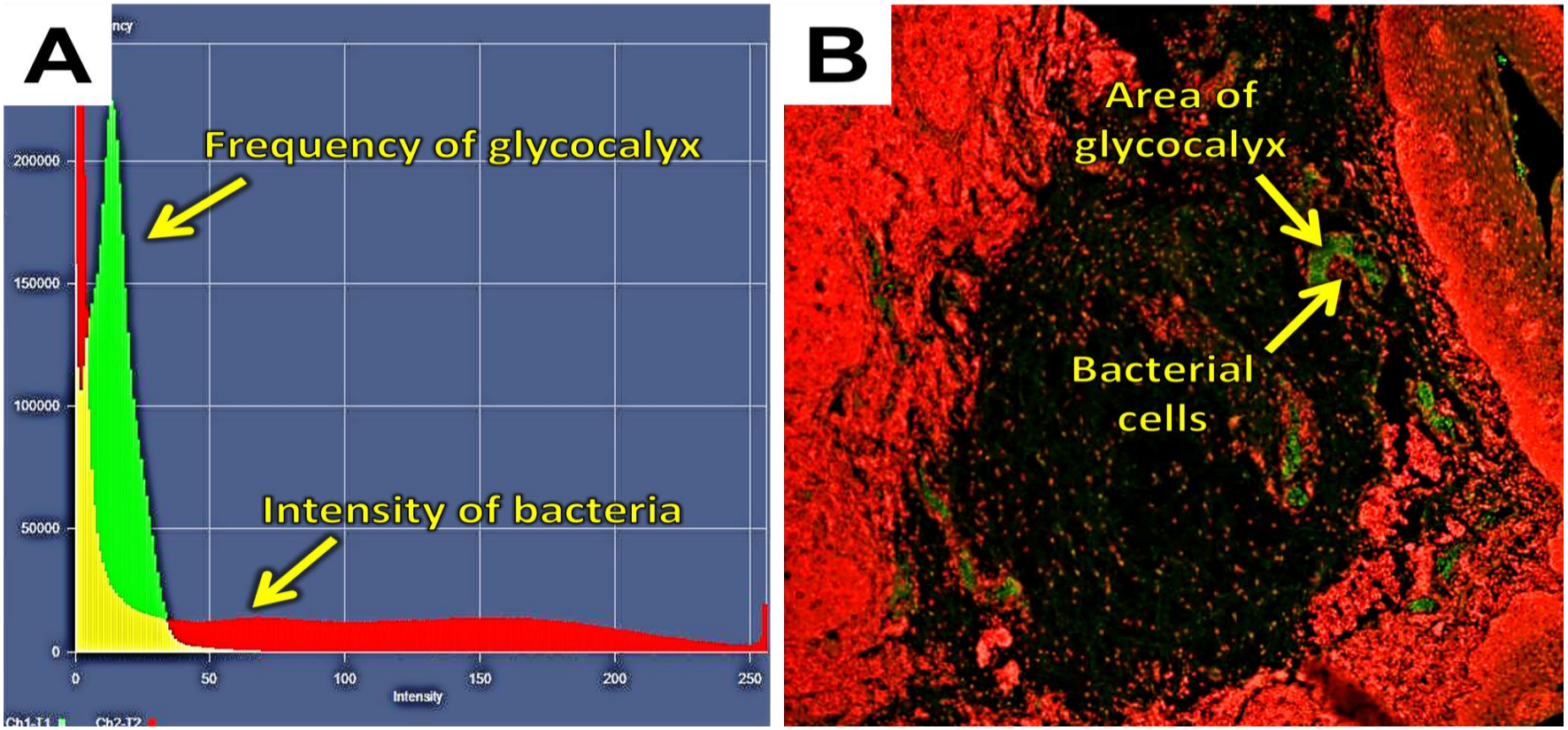

Figure 4.10 Evidence of fluorescent-labeled structures from a representative biofilm image via CLSM. **A,** Histometric measurement of the detected biofilm indicating absolute frequency of glycocalyx (green) in comparison with intensity of bacterial cells (red). **B,** Frequency of the detected glycocalyx (green) within the tonsillar crypt surrounded by tonsillar nuclei (red). Arrows indicate the biofilm structures and tissue sections were stained with Propidium iodide & Concanavalin A.

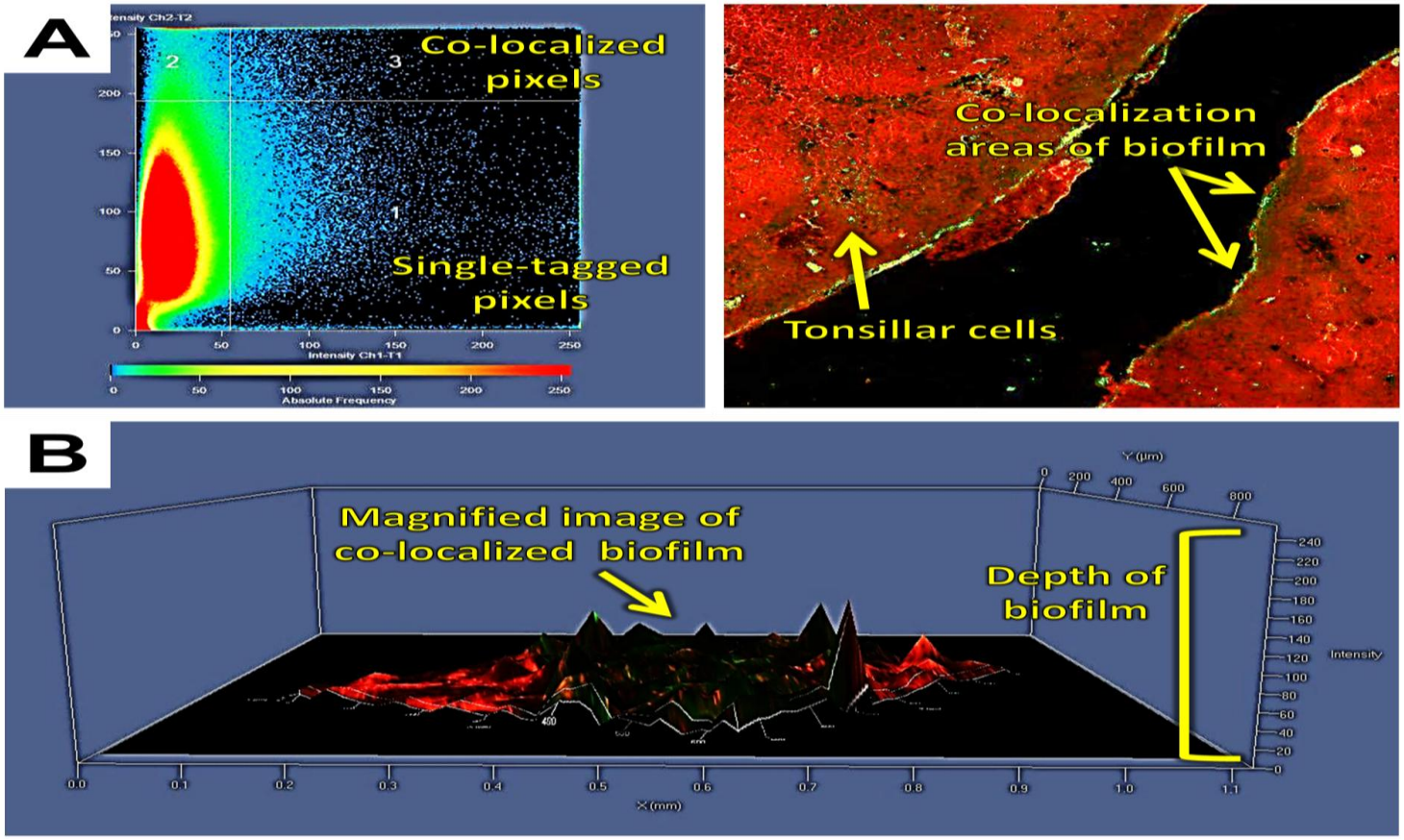

Figure 4.11 Evidence of co-localization areas from a representative biofilm image via CLSM. **A,** Co-localized areas of a biofilm within the space of tonsillar crypt (10x). **B,** Magnified 2.5-D image of a co-localized biofilm showing the depth of the structure. Arrows indicate the biofilm structures and tissue sections were stained with Propidium iodide & Concanavalin A.

Based on the data obtained from clinical examination for each patient, tonsillar hypertrophy was the most frequent symptom presented by adults and children. A significantly higher percentage of patients presented infection manifestation (sore throat) rather than obstruction (snore, nasal obstruction, tonsillar hypertrophy, adenoids hypertrophy and apnea). Interestingly, the symptoms presented by each of the patients were associated with the presence of biofilms in the tonsils as examined microscopically via CLSM. An association between the most frequent clinical symptoms of and biofilm presence is shown (Table 4.3). Detailed table for the evidence of bacterial biofilms detected via CLSM among clinical cases is described (Appendix 10).

Table 4.3 Association between clinical symptoms and biofilm presence in the tonsils.

Clinical Symptom	Patients with Evidence of Biofilm
1. Tonsillar hypertrophy	42 (60%) *
2. Sore throat	40 (57%) *
3. Adenoid hypertrophy	13 (18%) *
4. Apnea	10 (14%) *
5. Nasal obstruction	10 (14%) *

*** Percentage was calculated based on the total number of patients which was 70**

4.6 Biofilm Formation Ability

4.6.1 Microtiter Plate Assay

The MTP assay allowed us to quantify the rate of adherence and subsequent biofilm formation of tested bacteria. The ability of biofilm formation appeared to be

related to the type of isolate rather than the site of isolation, in this case, a significant increase in the adherence index was detected spectrophotometrically among *Staphylococcus aureus*, *Haemophilus influenzae*, GABHS, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* strains considering them as strong biofilm formers. However, a weak biofilm formation abilitiy indicated by a decrease in the value of adherence index was detetcd among *Streptococcus agalacticae*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, Group C Streptococci and *Acinetobacter baumannii*. Whereas no biofilm formation abilitiy was detected among Group C Streptococci, *Citrobacter* and *Enterobacter cloacae.*

Among our 464 tonsillar isolates, the number and percentages of biofilm formers were 78 (42.39%) isolates of *S. aureus*, 55 (63.95%) isolates of *H. influenzae*, 19 (33.92%) isolates of *Streptococcus agalactiae (*Group B Streptococci), 16 (51.61%) isolates of *H. parainfluenzae*, 15 (50%) isolates of *K. pneumoniae*, 6 (24%) isolates of Group G Streptococci (GGS), 12 (85.71%) isolates of GABHS, 7 (77.77%) isolates of *P. aeruginosa*, 2 (25%) isolates of Group C Streptococci (GCS) and 2 (66.66%) isolates of *S. pneumoniae*. Quantification of biofilm formation based on the adherence index among all the tonsillar clinical isolates is illustrated in (Figure 4.12). Moreover, there was a strong correlation between the presence of biofilms as detected microscopically in tonsillar tissue sections and the ability of tonsillar clinical isolates to form biofilm via MPT assay.

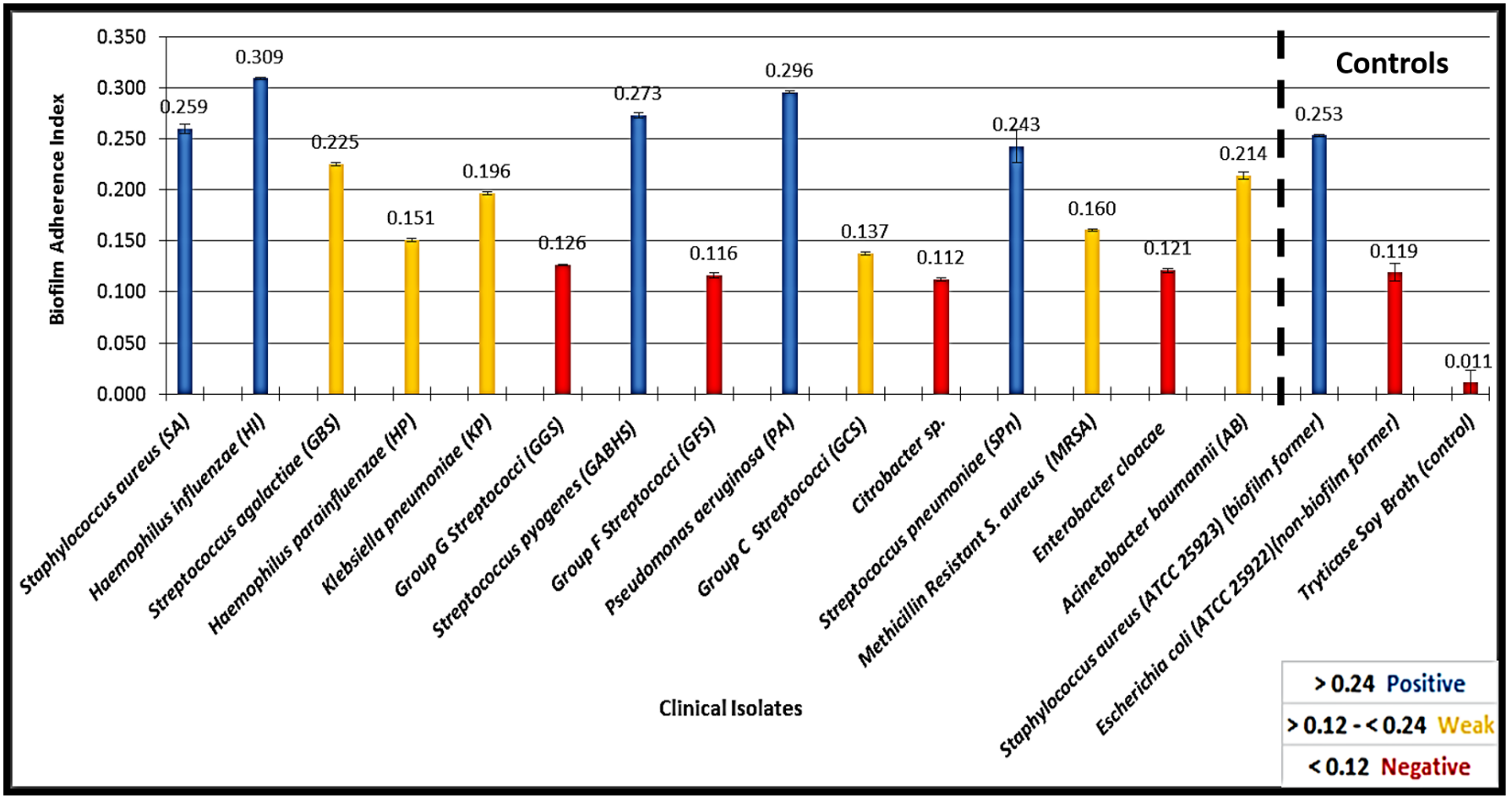

Figure 4.12 Biofilm formation ability among tonsillar clinical isolates via MTP assay. Positive result indicates a strong biofilm formation ability whereas negative result indicates no biofilm formation.

4.6.2 Congo Red Agar Method

By examining the morphology of colonies on Congo Red Agar for each of our bacterial isolates, it was possible to define eight major morphological variations (colonial morphotypes) as shown in (Figure 4.13). The biofilm formation morphotypes had 4 variations including very black (VB) colonies (Figure 4.13 [1]) indicating strong slime production, Bright Black (BB) colonies (Figure 4.13 [2]) indicating strong slime production, Almost Black (AB) colonies (Figure 4.13 [3]) indicating moderate slime production and Dry Black (DB) colonies indicating weak slime production (Figure 4.13 [4]). However, the non-biofilm forming morphotypes had also 4 variations where all indicated no virulence or no production of slime. These include the Orange (OR) colonies (Figure 4.13 [5]), Pinkish Red (PR) colonies (Figure 4.13 [6]), Bordeaux (BR) colonies (Figure 4.13 [7]) and Very Red (VR) colonies (Figure 4.13 [8]).

Although it was possible to classify most variants as one of the major morphotypes, there were some intermediate forms which were difficult to categorize. For example in the Bordeaux (BR) phenotype had a great tendency to undergo colonial variation more than the rest. Moreover, dryness of the agar or heavy inoculation of plates sometimes resulted in altered morphology. Another type of colonial variation was sometimes observed when CRA plates remained at room temperature for several days beyond the standard 2 day incubation period. Variants appeared as crops of tiny pink colonies imposed on the primary inoculum. However, subculture of these forms yielded normal sized colonies belonging to the original morphotype. It should be noted that CRA was used in this study to facilitate the detection of variant forms in correlation with the results obtained from MPT assay.

Based on CRA results, out of the 464 isolates, three main categories can be determined. Firstly, invasive bacteria comprising of 5 types that were regarded as strong biofilm formers including *Haemophilus influenzae* with the highest mean of slime

production followed by *Pseudomonas aeruginosa*, GABHS, *Staphylococcus aureus* then *Streptococcus pneumoniae*. Secondly, colonizing bacteria were comprised of 6 types that were regarded as weak biofilm formers including *Streptococcus agalactiae* with a moderate mean of slime production followed by *Klebsiella pneumoniae*, *Haemophilus parainfluenzae* and Group C Streptococci. Thirdly, commensal bacteria comprising of 4 types that were regarded as non-biofilm formers including Group G Streptococci, Group F Streptococci, *Citrobacter* sp. and *Enterobacter cloacae*.

The CRA method allows the formation of colonies on the plate to be directly monitored for morphological variations. This is particularly significant because there is evidence to suggest that the phase variability itself should be regarded as an important virulence marker. The results of CRA were strongly associated with the results of MTP assay and there was no significance difference between the biofilm formation ability among tonsillar isolates (Table 4.4). Detailed tables for biofilm formation abilities among all clinical isolates using MPT assay, CRA method and microscopic observations is described (Appendixes 11A, 11B, 11C, 11D, 11E, 11F, 11G, 11H, 11I, 11J, 11K, 11L, 11M, 11N, 11O).

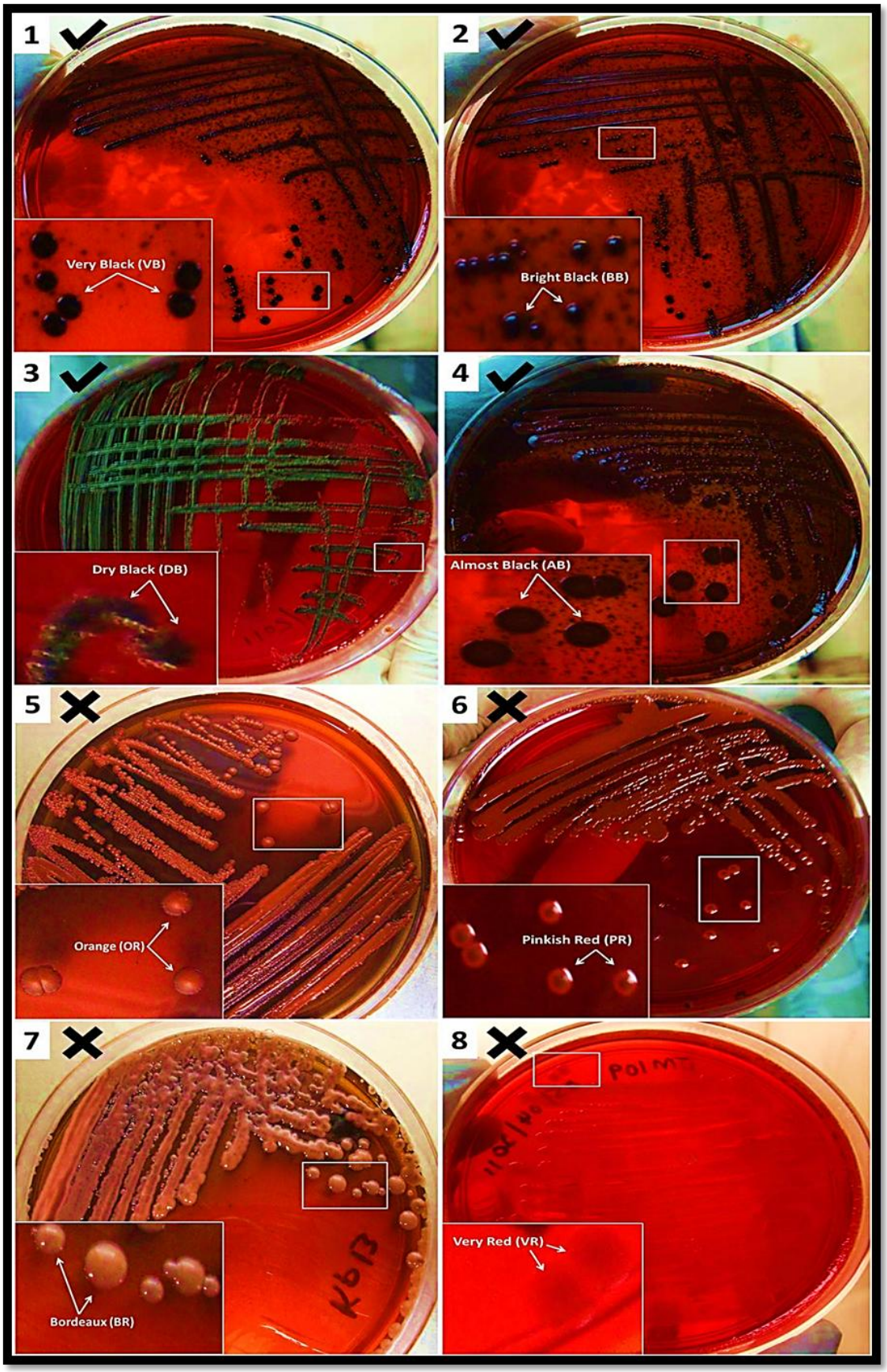

Figure 4.13 Morphological variations of bacterial colonies via CRA method. **1-4,** Indicates biofilm forming strains. **5-8,** Indicates non-biofilm forming strains. The "√" sign represent positive results (slime production) whereas "x" sign represent negative result (no slime production).

Table 4.4 Comparison of biofilm formation abilities among clinical isolates by MTP assay and CRA method.

Clinical Isolate (Total No.)	CRA method		MTP assay	
	Biofilm former isolate	Non-Biofilm former isolate	Biofilm former isolate	Non-Biofilm former isolate
Gram-Positive				
Methicillin Resistant *S. aureus* (1)	1	0	1	0
Staphylococcus aureus (184)	77	107	82	102
Streptococcus agalactiae (56)	21	35	25	31
Group F Streptococci (11)	4	7	4	7
Group C Streptococci (8)	2	6	3	5
Group G Streptococci (25)	9	16	10	15
Streptococcus pyogenes (14)	9	5	11	3
Streptococcus pneumoniae (3)	1	2	1	2
SUBTOTAL	124	178	137	165
Gram-Negative				
Acinetobacter baumannii (1)	1	0	0	1
Citrobacter sp. (4)	1	3	0	4
Enterobacter cloacae (1)	0	1	0	1
Haemophilus influenzae (86)	45	41	53	33
Haemophilus parainfluenzae (31)	14	17	18	13
Klebsiella pneumoniae (30)	15	15	17	13
Pseudomonas aeruginosa (9)	7	2	7	2
SUBTOTAL	83	79	95	67
TOTAL	207	257	232	232

4.7 Morphology Confirmation of *Paenibacillus haemolyticus* strain 139SI

The morphological confirmation to identify our isolate as *Paenibacillus haemolyticus* strain 139SI showed that cells of the strains were Gram-positive rod-shaped with bipolar end terminal spores (Figure 4.13). Moreover, colonies of the isolate when grown on Colombia agar supplemented with 5% sheep blood were grey, large, rough and irregular edged with a size of 2–3 mm in diameter exhibiting a strong haemolytic activity after 16 h incubation at 37◦C (Figure 4.14). Conformation of the cellular morphology via SEM showed all cells regular rods with a size of 0.4–0.5 µm ×

2.0–2.5 µm (Figure 4.15). Our report to discover the first haemolytic *Paenibacillus haemolyticus* with growth promoting activities of this work was published at Biologia (Appendix 3B). The strain was deposited with the American Type Culture Collection (ATCC) (Appendix 6) and its 16SrRNA sequence was assigned an accession number at GenBank (Appendix 7).

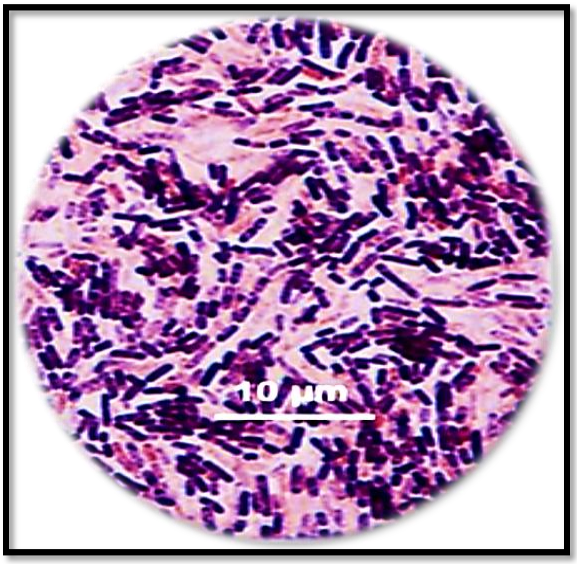

Figure 4.14 Light Microscopy image showing Gram-positive bacilli cells of *Paenibacillus haemolyticus* strain 139SI when grown on BHI broth after 48 h incubation at 37^{o}C. Image viewed under LM (100x).

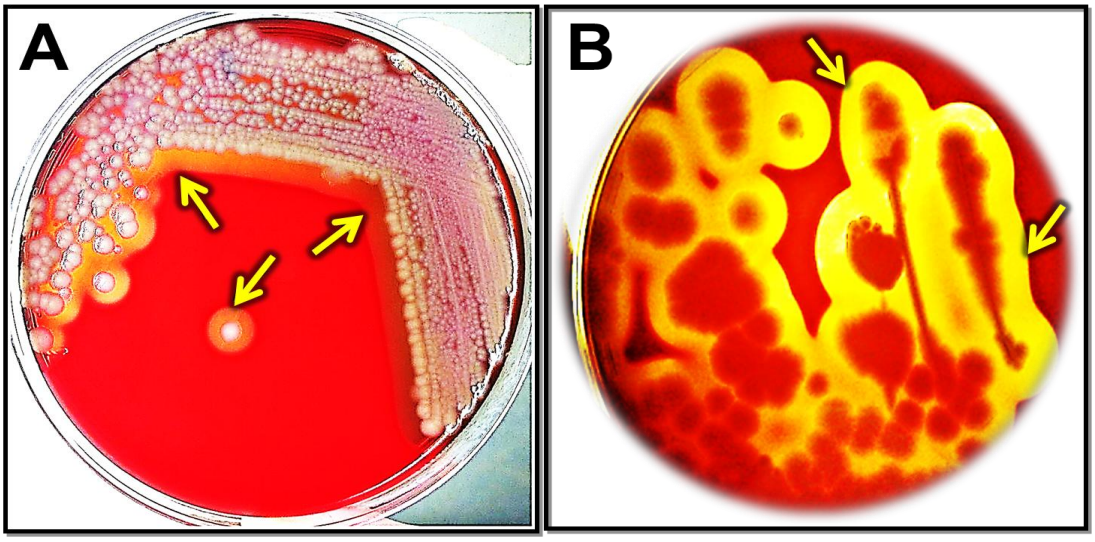

Figure 4.15 Colony morphology of the novel soil bacteria *Paenibacillus haemolyticus* strain 139SI grown on Columbia agar supplemented with 5% sheep blood. **A,** Cells after 24 h incubation at 37^{o}C. **B,** Cells after 48 h incubation at 37^{o}C. Arrows indicate the complete hemolysis of blood in the medium (β-hemolysis).

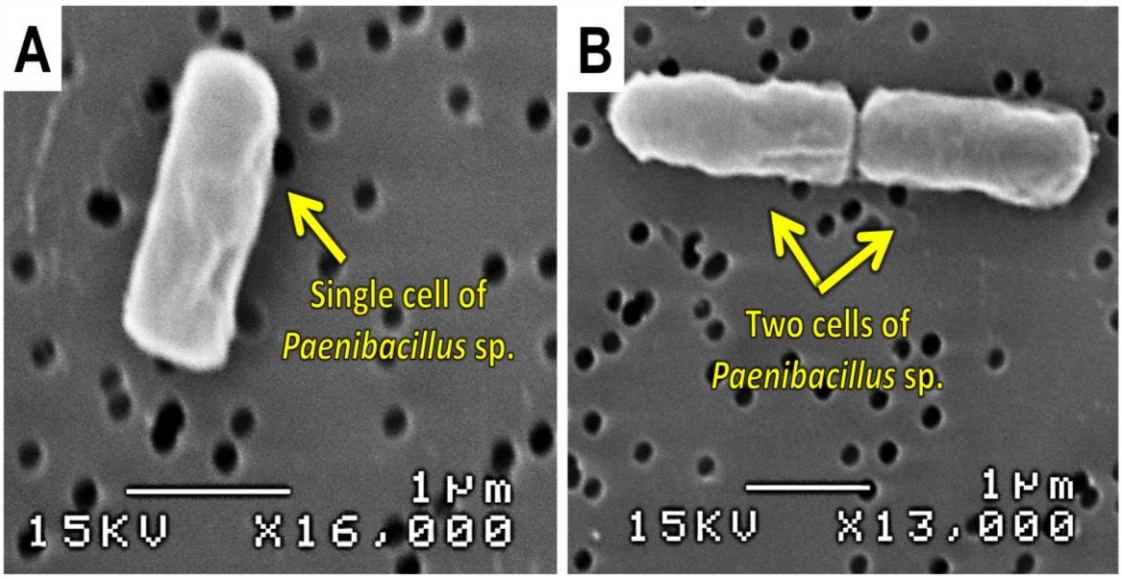

Figure 4.16 Cellular morphology of *Paenibacillus haemolyticus* strain 139SI as viewed by SEM. The image shows the strain when grown on BHI broth after 48 h incubation at 37^{o}C. **A,** Single regular bacilli cells with a size of 0.4–0.8 × 2–5 µm. (16000x). **B,** Two multiplying cells (13000x). Arrows indicates cells.

4.8 *In vitro* Antibiofilm Activity of 139SI Culture Filtrate

4.8.1 MPT Assay

The results of MTP showed significant inhibition of biofilm formation among selected Gram-positive tonsillar isolates represented by *Staphylococcus aureus*, *Streptococcus agalactiae*, Group G Streptococci, *Streptococcus pyogenes* and *Streptococcus pneumoniae*. In addition to the Gram-negative isolates represented by *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Citrobacter* sp. and *Pseudomonas aeruginosa*. The most effective concentration was 4500 µg/ml that resulted in a decreased adherence index when quantified spectrophotometrically (Table 4.5 and Table 4.6). There was a significant decrease in the adherence index among all the isolates tested with variations in the degree of adherence to the surface.

Table 4.5 Antibiofilm activity of 139SI filtrate against Gram-negative isolates. The lowest most active concentration is highlighted.

139SI Filtrate Concentration	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Citrobacter sp.*	Reference strain *P. aeruginosa* ATCC 27853
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
2500 µg/ml	0.277 ± 0.002	0.376 ± 0.060	0.214 ± 0.030	0.216 ± 0.073	0.257 ± 0.003	0.124 ± 0.003
3000 µg/ml	0.277 ± 0.002	0.376 ± 0.060	0.210 ± 0.024	0.206 ± 0.055	0.257 ± 0.003	0.245 ± 0.003
3500 µg/ml	0.225 ± 0.007	0.326 ± 0.020	0.224 ± 0.005	0.202 ± 0.099	0.216 ± 0.005	0.121 ± 0.001
4000 µg/ml	0.210 ± 0.003	0.285 ± 0.058	0.176 ± 0.004	0.174 ± 0.083	0.163 ± 0.001	0.204 ± 0.003
4500 µg/ml	0.211 ± 0.002	0.244 ± 0.113	0.149 ± 0.009	0.149 ± 0.057	0.157 ± 0.003	0.225 ± 0.004
2(5H)-Furanone (Positive Control)	0.083 ± 0.005	0.092 ± 0.004	0.093 ± 0.003	0.100 ± 0.001	0.110 ± 0.010	0.127 ± 0.003
BHI broth (Nagtive Control)	0.044 ± 0.011	0.038 ± 0.003	0.060 ± 0.002	0.074 ± 0.003	0.055 ± 0.041	0.035 ± 0.007

OD = Optical Density, SD = Standard Deviation, BHI = Brain Heart Infusion

Table 4.6 Antibiofilm activity of 139SI filtrate against Gram-positive isolates. The lowest most active concentration is highlighted.

139SI Filtrate Concentration	*Staphylococcus aureus*	*Streptococcus agalactiae*	Group G Streptococci	*Streptococcus pyogenes*	*Streptococcus pneumoniae*	Reference strain *S. aureus* ATCC 25923
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
2500 µg/ml	0.486 ± 0.360	0.329 ± 0.113	0.363 ± 0.079	0.377 ± 0.122	0.404 ± 0.003	0.254 ± 0.004
3000 µg/ml	0.276 ± 0.004	0.232 ± 0.115	0.257 ± 0.024	0.309 ± 0.114	0.368 ± 0.028	0.254 ± 0.004
3500 µg/ml	0.270 ± 0.042	0.208 ± 0.074	0.243 ± 0.002	2.76 ± 0.056	0.368 ± 0.028	0.246 ± 0.004
4000 µg/ml	0.270 ± 0.027	0.170 ± 0.024	0.157 ± 0.007	0.316 ± 0.056	0.324 ± 0.003	0.232 ± 0.005
4500 µg/ml	0.243 ± 0.045	0.142 ± 0.003	0.157 ± 0.007	0.262 ± 0.003	0.254 ± 0.003	0.188 ± 0.005
2(5H)-Furanone (Positive Control)	0.082 ± 0.016	0.100 ± 0.085	0.091 ± 0.006	0.082 ± 0.006	0.100 ± 0.005	0.113 ± 0.007
BHI broth (Nagtive Control)	0.057 ± 0.038	0.050 ± 0.006	0.021 ± 0.002	0.069 ± 0.020	0.047 ± 0.005	0.053 ± 0.004

OD = Optical Density, SD = Standard Deviation, BHI = Brain Heart Infusion

4.8.2 BIC Test

The BIC test was performed against selected *Pseudomonas aerugniosa* isolates as this species will be used later in the biofilm model of chronic lung infection. The139SI filtrate inhibited the bacterial attachment of *P. aeruginosa* when grown on the bottom of 6-well microtiter plate (Figures 4.17). The lowest and most effective concentration to achieve this inhibition was found to be 4500 µg/ml and the biofilms were inhibited up to 80% when visualized microscopically via LM and SEM (Figure 4.18) and (Figure 4.19) respectively.

Microscopic observation of Biofilm Inhibitory Concentration test via SEM showed that the biofilm of *P. aeruginosa* isolate coating the plastic surface of microtitter plate was disrupted after exposure to our soil bacterial culture filtrate at concentration of 4500 µg/ml. (Figure 4.19) The remains of biofilm showing scattered bacterial cells and aggregations of bacterial cells with no extracellular matrix connecting them. However, exposure to the 139SI filtrate at a lower consternation, i.e. 3000 µg/ml, did not completely inhibit the biofilm of *P. aeruginosa* as indicated by the visualization of bacterial cells embedded in the remains of an amorphous extracellular matrix.

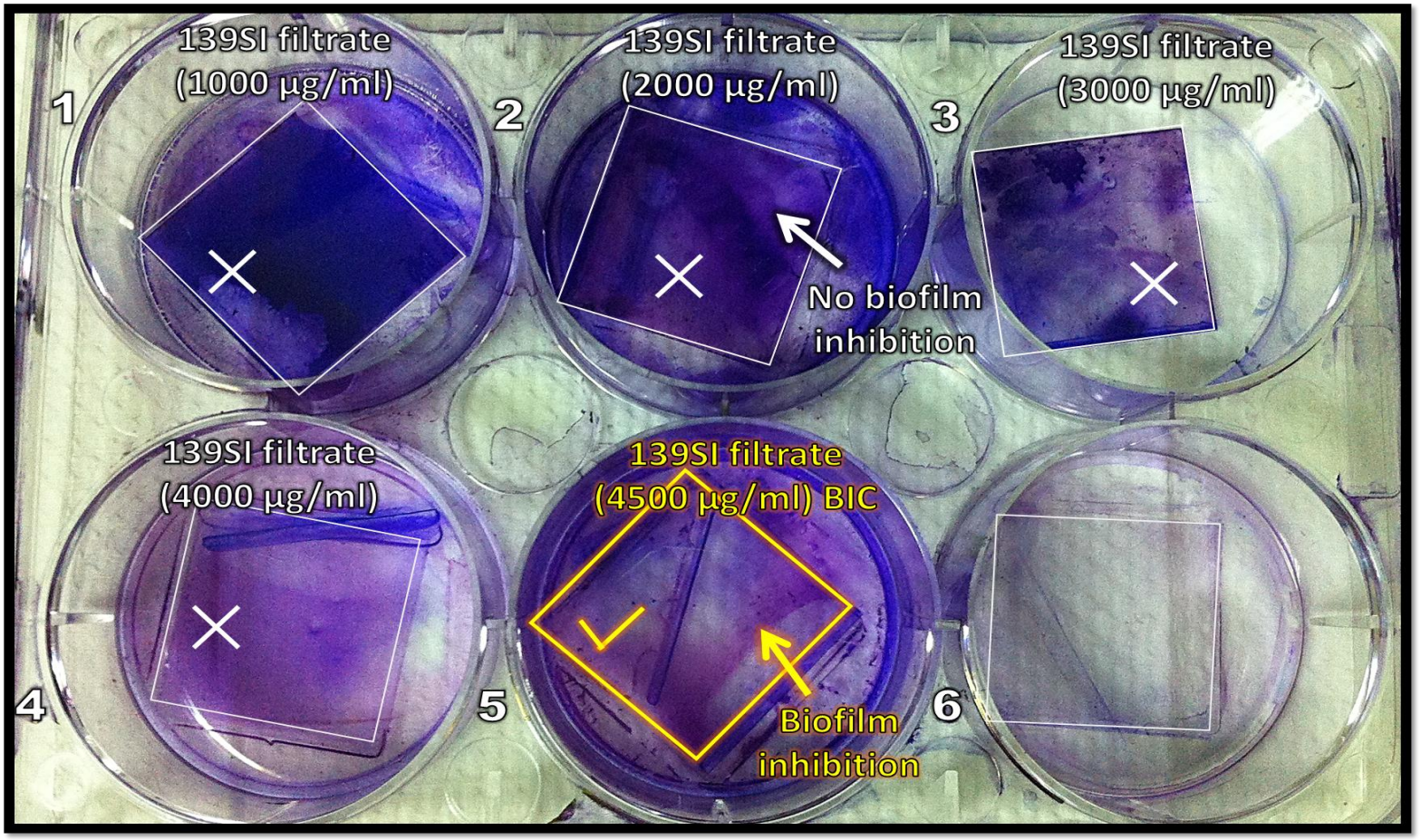

Figure 4.17 Biofilm Inhibitory Concentration (BIC) test of 139SI filtrate against *P. aeruginosa* biofilms that are attached to the surface of square cover slip placed inside the wells (1-6) of microtiter plate. The BIC of 139SI filtrate (4500 µg/ml) is highlighted and "x" sign indicates no or partial biofilm inhibition whereas "√" sign indicates complete biofilm inhibition.

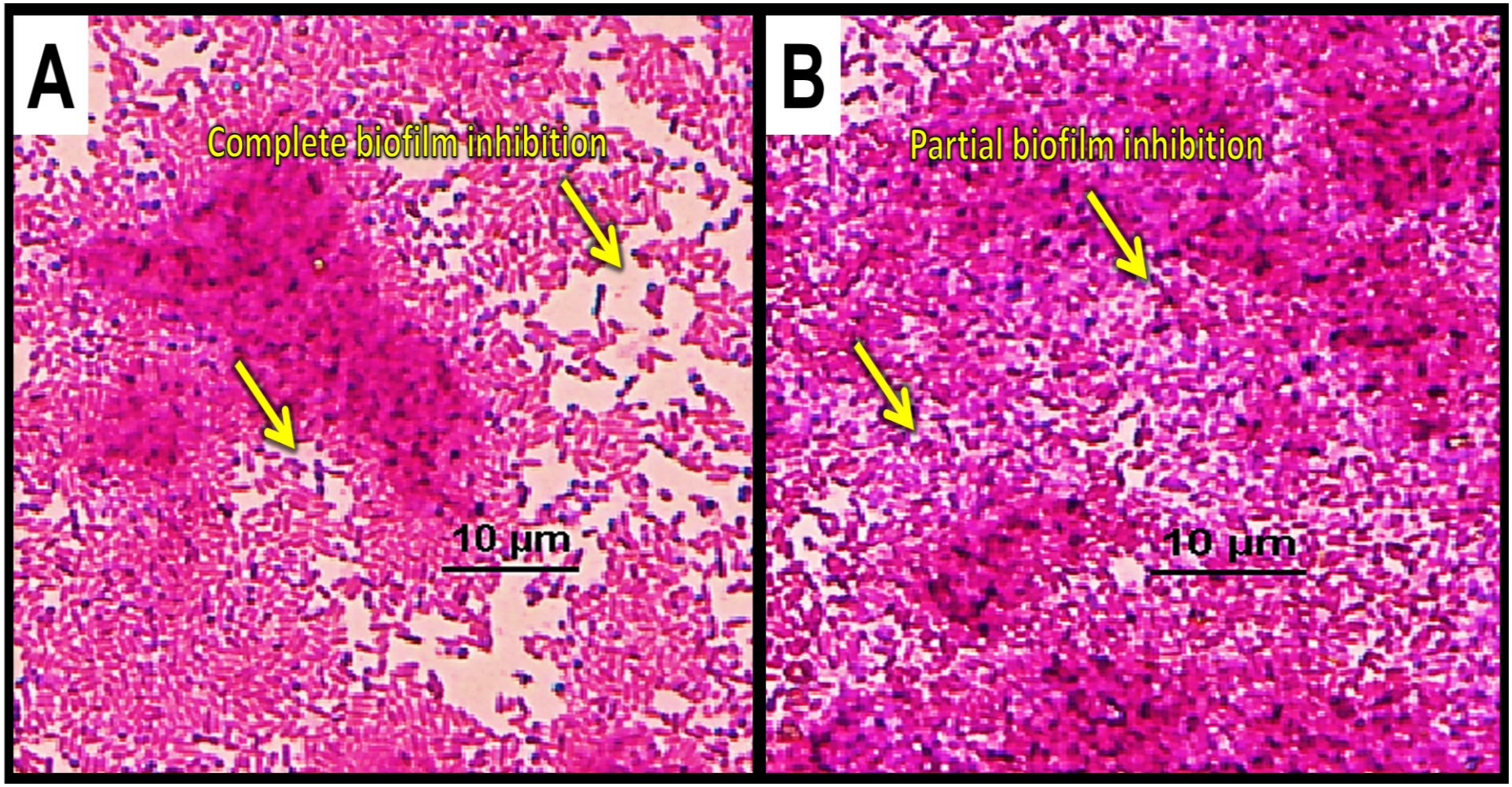

Figure 4.18 Microscopic observation of Biofilm Inhibitory Concentration via LM against biofilms of *P. aeruginosa* coating the glass cover slip and stained with Crystal violate. **A,** Complete inhibition of biofilm after exposure to 139SI filtrate at BIC (4500 µg/ml). **B,** Partial inhibition of biofilm after exposure to 139SI filtrate at non-BIC (4000 µg/ml). Arrows indicate the sites of biofilm inhibition.

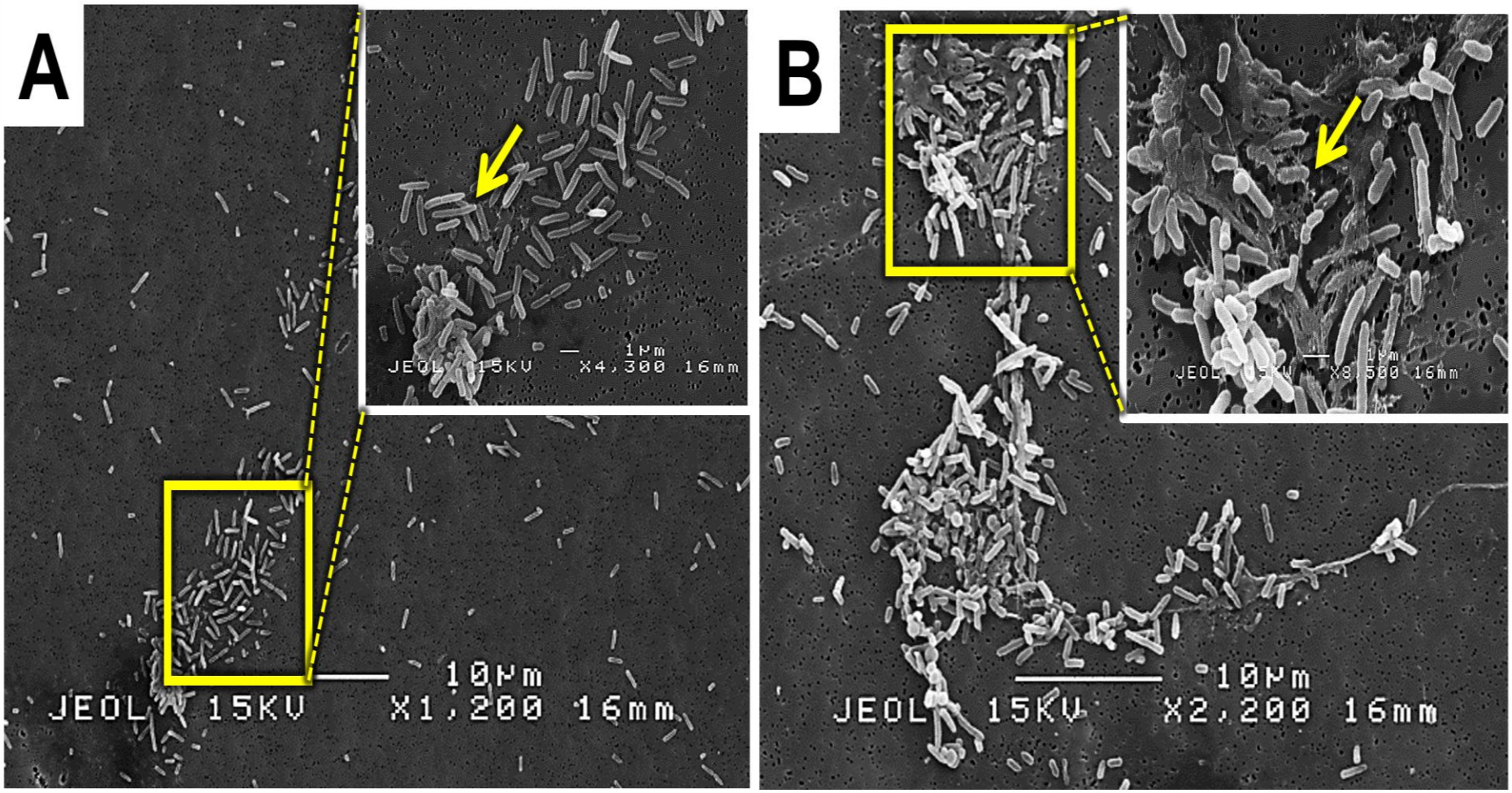

Figure 4.19 Microscopic observation of Biofilm Inhibitory Concentration of via SEM against *P. aeruginosa* biofilms coating the 6-well of microtiter plate. **A,** Complete biofilm inhibition after exposure to 139SI filtrate at BIC concentration (4500 µg/ml). **D,** Partial biofilm inhibition after exposure to 139SI filtrate at non-BIC concentration (4000 µg/ml). Arrows indicates sites of biofilm inhibition.

4.9 Acute Toxicity Test of 139SI Culture Filtrate

4.9.1 Gross General Observations

The rats were treated with low and high dose of 139SI filtrate while the control group was treated with 0.9% normal saline. Gross general observations indicated that all experimental rats showed no signs of tremor, convulsions and reflex abnormalities. No muscular numbness, salivation and diarrhea were observed. The food intake per day was also being found normal. The average and individual body weights of all rats were increased after the oral administration of 139SI filtrate which was statistically insignificant. From these observations we can conclude that the isolated compound has no adverse effect on normal growth of SD rats.

4.9.2 Biochemical Parameters and Haematological Profiles

The biochemical parameters changed slightly however remained within the normal range for both males and females except for few slight abnormalities as shown in Tables 4.7, 4.8, 4.9 and 4.10. All analyte values were expressed as the mean ± S.E.M. with $p<0.05$ as the significant value. There are no statistically significant differences between experimental groups which indicates the non-toxic nature of 139SI filtrate on both kidney and liver functions. The haematological profile was studied and the oral administrations of 139SI filtrate to check the haematological disorders. No abnormalities were found in the total count of white blood cells (WBCs), red blood cells (RBCs), platelet count and haemoglobin percentage of both low dose (2 gm/kg) and low does (4 gm/kg) in comparison with the control (vehicle) group. These data indicates that139SI filtrate has no substantial effect on the haematological structure.

Table 4.7 Renal function test results among male rats.

Renal Function Test (Analyte)	Male Experimental Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 g/kg)	High Dose (5 g/kg)		
Sodium	139.00 ± 1.12	138.66 ± 0.91	137.83 ± 1.40	136 - 145	mmol/L
Potassium	*6.93 ± 0.79	*6.03 ± 0.25	*6.45 ± 0.72	3.6 - 5.2	mmol/L
Chloride	101.83 ± 1.01	102.00 ± 1.03	102.16 ± 1.24	100 - 108	mmol/L
Carbon Dioxide	28.05 ± 1.50	29.21 ± 0.72	26.41 ± 1.38	21.0 - 30.0	mmol/L
Anion Gap	16.16 ± 1.37	14.76 ± 0.74	16.76 ± 2.11	10 - 20	mmol/L
Urea	*7.78 ± 0.81	*8.71 ± 0.71	*9.35 ± 1.77	2.5 - 6.4	mmol/L
Creatinine	*18.16 ± 2.38	*21.00 ± 3.29	*20.00 ± 4.67	61.9 - 115	µmol/L

mmol/L = millimole per Liter, µmol/L = micromole per Liter
* indicates values that are above or below the reference range

Table 4.8 Renal function test results among female rats.

Renal Function Test (Analyte)	Female Experiment Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 g/kg)	High Dose (5 g/kg)		
Sodium	139.50 ± 0.99	138.00 ± 1.06	138.50 ± 0.84	136 - 145	mmol/L
Potassium	*6.80 ± 0.59	*6.10 ± 0.65	*5.56 ± 0.25	3.6 - 5.2	mmol/L
Chloride	96.66 ± 4.70	100.66 ± 1.05	98.50 ± 2.61	100 - 108	mmol/L
Carbon Dioxide	24.68 ± 1.04	27.03 ± 1.35	26.66 ± 1.37	21.0 - 30.0	mmol/L
Anion Gap	*24.68 ± 1.04	*27.03 ± 1.35	*26.66 ± 1.37	10 - 20	mmol/L
Urea	*7.50 ± 1.12	*7.15 ± 0.32	*7.23 ± 0.64	2.5 - 6.4	mmol/L
Creatinine	*23.33 ± 2.76	*24.50 ± 3.78	*28.66 ± 1.20	61.9 - 115	µmol/L

mmol/L = millimole per Liter, µmol/L = micromole per Liter
* indicates values that are above or below the reference range

Table 4.9 Liver function test results among male rats.

Liver Function Test (Analyte)	Male Experiment Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 g/kg)	High Dose (5 g/kg)		
Total Protein	57.50 ± 2.45	67.33 ± 1.72	65.83 ± 2.79	64 - 82	g/L
Albumin	10.00 ± 2.04	12.46 ± 1.35	16.31 ± 5.98	35 - 50	g/L
Globulin	*42.33 ± 4.77	54.33 ± 1.83	50.83 ± 4.14	23 - 35	g/L
Total Bilirubin	3.66 ± 0.76	0.31 ± 0.47	3.75 ± 1.12	3 - 17	µmol/L
Conjugate Bilirubin	1.50 ± 0.34	1.33 ± 0.21	1.50 ± 0.34	0 - 3	µmol/L
Alkaline Phosphatase	*256.16 ± 29.30	229.66 ± 22.48	195.50 ± 22.10	50 - 136	IU/L
Alanine Aminotransferase	*74.50 ± 10.59	83.16 ± 5.67	107.16 ± 16.66	30 - 65	IU/L
Aspartate Aminotransferase	*210.50 ± 22.81	188.16 ± 20.13	201.16 ± 17.64	15 - 37	IU/L
G-Glutamyltransferase	*11.00 ± 3.35	8.33 ± 2.06	10.33 ± 4.27	15 - 85	IU/L

µmol/L = micromole per Liter, g/L = gram per Liter, IU/L = International Unit per Liter
* indicates values that are above or below the reference range

Table 4.10 Liver function test results among female rats.

Liver Function Test (Analyte)	Female Experiment Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 g/kg)	High Dose (5 g/kg)		
Total Protein	61.00 ± 3.81	71.66 ± 0.95	63.16 ± 2.46	64 - 82	g/L
Albumin	15.83 ± 3.00	13.66 ± 1.02	16.66 ± 5.74	35 - 50	g/L
Globulin	*45.83 ± 3.77	57.50 ± 1.64	48.16 ± 4.85	23 - 35	g/L
Total Bilirubin	6.83 ± 2.15	2.83 ± 0.83	4.66 ± 1.76	3 - 17	µmol/L
Conjugate Bilirubin	1.83 ± 0.40	1.33 ± 0.33	1.50 ± 0.34	0 - 3	µmol/L
Alkaline Phosphatase	*156.33 ± 38.10	136.50 ± 21.53	140.00 ± 23.42	50 - 136	IU/L
Alanine Aminotransferase	*67.50 ± 11.44	67.50 ± 3.19	82.33 ± 16.32	30 - 65	IU/L
Aspartate Aminotransferase	*150.33 ± 19.10	196.66 ± 19.69	196.83 ± 24.25	15 - 37	IU/L
G-Glutamyltransferase	*5.66 ± 0.71	4.50 ± 0.56	7.66 ± 1.68	15 - 85	IU/L

µmol/L = micromole per Liter, g/L = gram per Liter, IU/L = International Unit per Liter
* indicates values that are above or below the reference range

4.9.3 Histopathological Evaluation

An evaluation of the histopathological (toxicological) effects of our 139SI bacterial culture filtrate was carried out by examining the liver and kidney of all experimental groups both males and females. No significant differences were detected in the histopathology of these organs whether among high or low doses groups as well the vehicle group. These findings indicate that the compound from 139SI filtrate that contains secondary metabolites have no effects on cellular structures meaning that they do not cause degeneration of the cells of these organs (Figure 4.20).

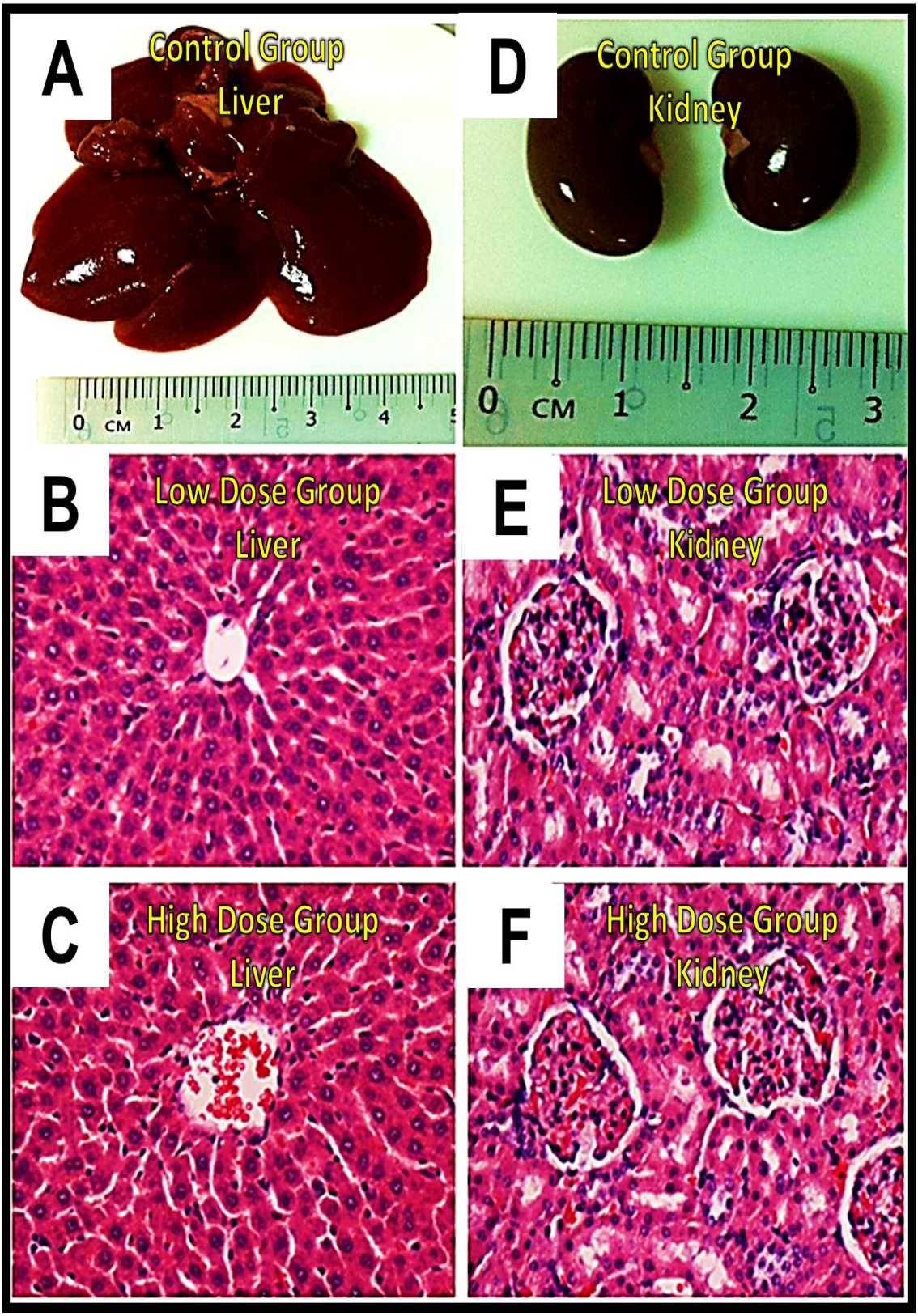

Figure 4.20 Gross and histopathology evaluation of liver and kidney in experimental groups based on acute toxicity test. **A,** Normal liver gross among vehicle (control) group. **B,** Normal liver histology among low dose group. **C,** Normal liver histology among high dose group. **D,** Normal kidney gross among vehicle (control) group. **E,** Normal kidney histology among low dose group. **F,** Normal kideny histology among high dose group. Tissue sections were stained with H&E.

4.10 *In vivo* Antibiofilm Activity of 139SI Culture Filtrate

Based on our results, we examined the therapeutic (treatment) effects of 139SI bacterial culture filtrate on the biofilm infection caused by a resistant strain of *P. aeruginosa* clinical isolate. The therapeutic effects were evaluated based on the daily oral administration of a low dose (25 gm/kg) culture filtrate that is believed to contain bioactive metabolites produced from a novel soil bacterial species, *Paenibacillus haemolyticus* strain 139SI. These effects included the survival time of rats, clearance of bacteria from the lungs, macroscopic and microscopic lung pathology. There was no mortality among rats from the negative group. However, it was noticed that all rats belonging to positive group (infection group) died before completing the course of experiment. The mortality among that group was noticed on various days, some died from a severe infection on day 2 post inoculation whereas others died on days 3 and 5. On the other hand, the survival time for the other groups particularly treatment group was noticed to be longer although few mortalities still occurred 2 (16.6%) on day 3 and 7; however it was statistically insignificant. The comparative group had its share of mortalities with 3 (25%) rats dying on days 6 and 7.

4.10.1 Lung Index of Macroscopic Pathology

To calculate the LIMP, which was used as an indicator of the severity of the lung pathology, we measured the area of the lungs exhibiting pathological changes. The major pathological changes observed were lung consolidation, abscesses and haemorrhage. On day 7 after challenge with *P. aeruginosa*, 4 rats were randomly selected for histopathology examination. On days 7, lung adhesion was rarely found, while lung consolidation with haemorrhage and abscesses were the main pathological changes. However, from day 14 on lung abscesses and haemorrhage became

predominant particularly in the positive control group, The LIMP for the four groups is shown in (Figure 4.21).

The 139SI filtrate exhibited promising activity in disrupting the biofilm P. aeruginosa in the lungs of both comparative control and treatment group which resulted in bacterial clearance from the lung and reduction of the severity of lung pathology. Treatment with the 139SI metabolite filtrate significantly prolonged survival times in the treatment group which was similar to the results found in comparative control group. It was noticed that areas with pathologic changes in the treatment group were smaller than that in the positive control group. The number of rats with chronic inflammation in both the treatment group 6 (12.5%) and comparative control group 6 (12.5%) was significantly lower than that in the positive control group 12 (25%) as shown in (Table 4.11). the severity of chronic inflammation was reduced to half when both 139SI filtrate and 2(5H)-Furanone were administered.

Table 4.11 Microscopic pathology of rat lungs 14 days after challenge with *Pseudomonas aeruginosa*.

Experimental Groups (No. of Rats)	Lung Microscopic Pathology No. (%) of Rats				
	Chronic Inflammation	Score I	Score II	Score II	Score IV
Negative Control (12)	NA	12 (25%)	NA	NA	NA
Positive Control (12)	12 (25%)	NA	NA	2 (4.16%)	10 (20.83%)
Comparative Control (12)	6 (12.5%)	1 (2.08%)	5 (10.4%)	4 (8.33%)	2 (4.16%)
Treatment 139SI (12)	6 (12.5%)	NA	6 (12.5%)	5 (10.4%)	1 (2.08%)
TOTAL (48)	18 (37.5%)	13 (27.08%)	11 (22.91%)	11 (22.91%)	13 (27.08%)

NA = Not Available

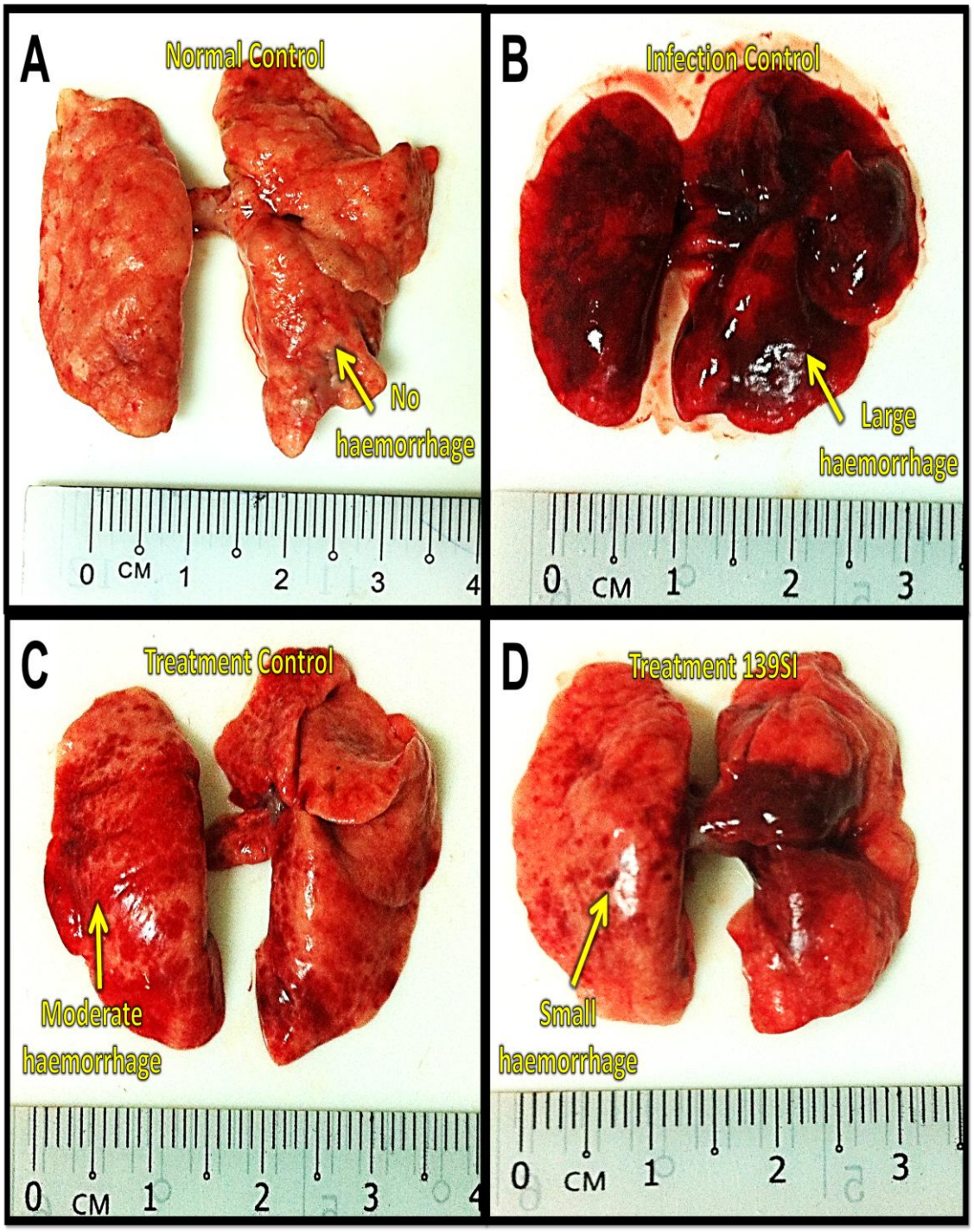

Figure 4.21 Gross pathology of lungs among experimental rats based on the lung index of macroscopic pathology. **A,** Normal lung showing no signs of haemorrhage. **B,** Infected lung with large haemorrhage and abscess (>40 mm^2). **C,** Swollen lungs with hyperemia, small atelectasis and moderate haemorrhage (10 mm^2 - 40 mm^2). **D,** Recovered lungs with hyperemia, atelectasis and moderate to small haemorrhage (<10 mm^2).

4.10.2 Histopathology Scoring of the Lungs

Upon random sacrifice of rats on day 3, the histopathology of lungs showed acute inflammation with an infiltration in which Polymorphonuclear leukocytes (PMNs) were predominant. Normal lungs were found in the negative control group with a score of I (Figure 4.22A) whereas chronic inflammation was found in positive (infection) control group showing severe inflammation to necrosis with a score of IV (Figure 4.22B). The treatment (139SI) group showed signs of recovery with a score of II (Figure 4.23A) whereas comparative control group showed moderate to severe focal inflammation with a score of III (Figure 4.23B). Furthermore, on day 14 the inflammation in positive control was significantly more severe compared to those of comparative and treatment groups. A score of IV in positive control group and a score of III in the treatment group was found on day 3. Areas in which the biofilm's alginate is attached and occupying the lung alveolar spaces are shown in (Figure 4.24). Moreover, to increase accuracy in detecting the therapeutic effects of 139SI filtrate, lung tissue sections were processed for immunohistochemistry and viewed under CLSM (Figure 4.25). The biofilm's glycocalyx was stained in green with Concanavalin A whereas the lung epithelium (alveolar cells) and the infecting bacteria were stained red with Propidium iodide. Comparative control tissue showed significant infiltration of PMNs in the lung whereas the infection control was surrounded by numerous PMNs. In the treatment group, inflammation was much milder and the infiltration was predominantly with mononuclear cells (MNs). The area with pathological changes in the treatment group were smaller than in comparative group. The incidence of acute inflammation in treatment group was lower than comparative control, but the difference was not statistically significant. Evidence of *P. aeruginosa* embedded in alginate and covering the lung's alveolar space are shown in (Figure 4.24 C and D).

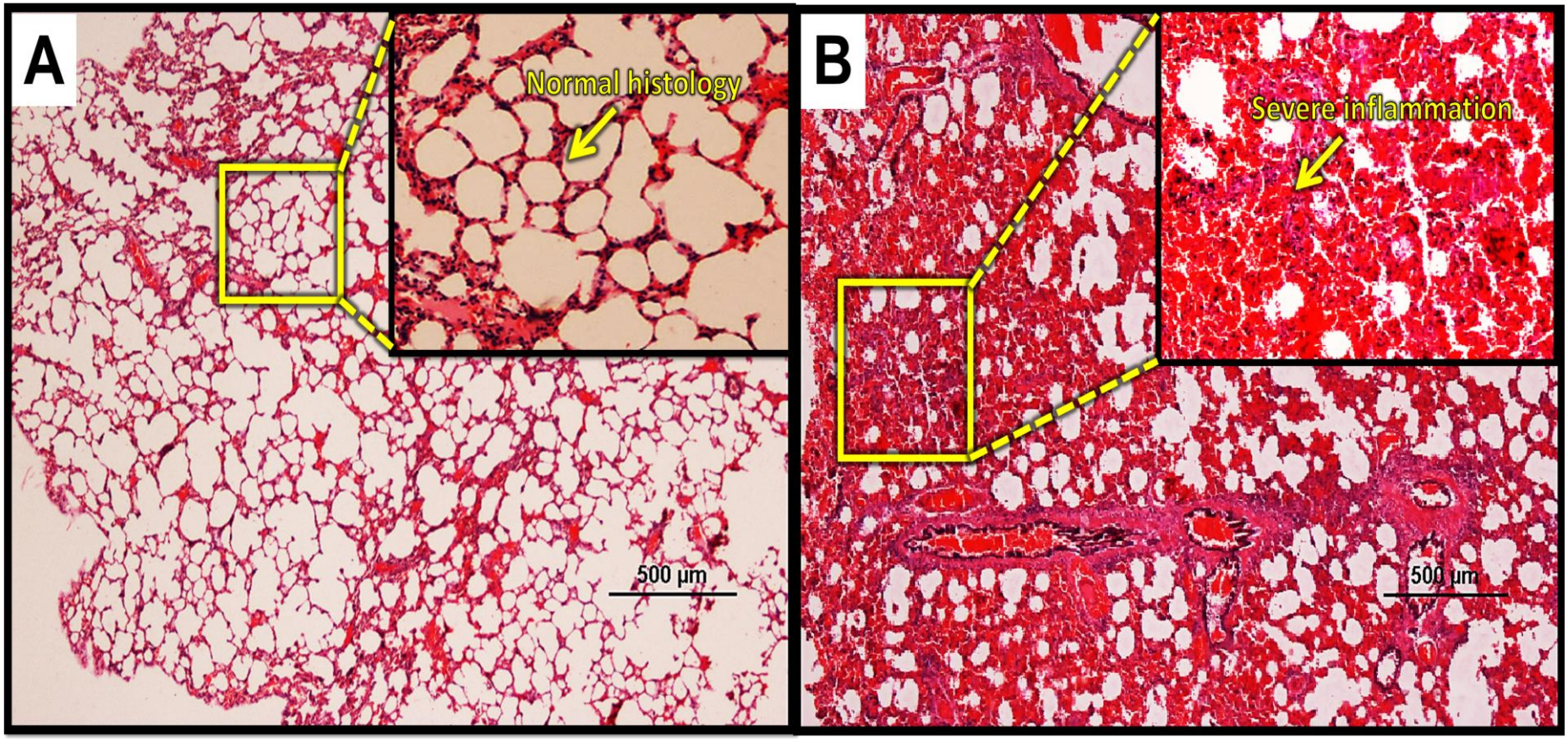

Figure 4.22 Histopathological scoring of lungs according to the severity of inflammation (Scores I and IV). **A,** Score I: with normal histology of the lung (negative control). **B,** Score IV with severe inflammation to necrosis throughout the lung (positive control) Low magnification 4x Arrows indicate the score of tissue and sections were stained with H&E.

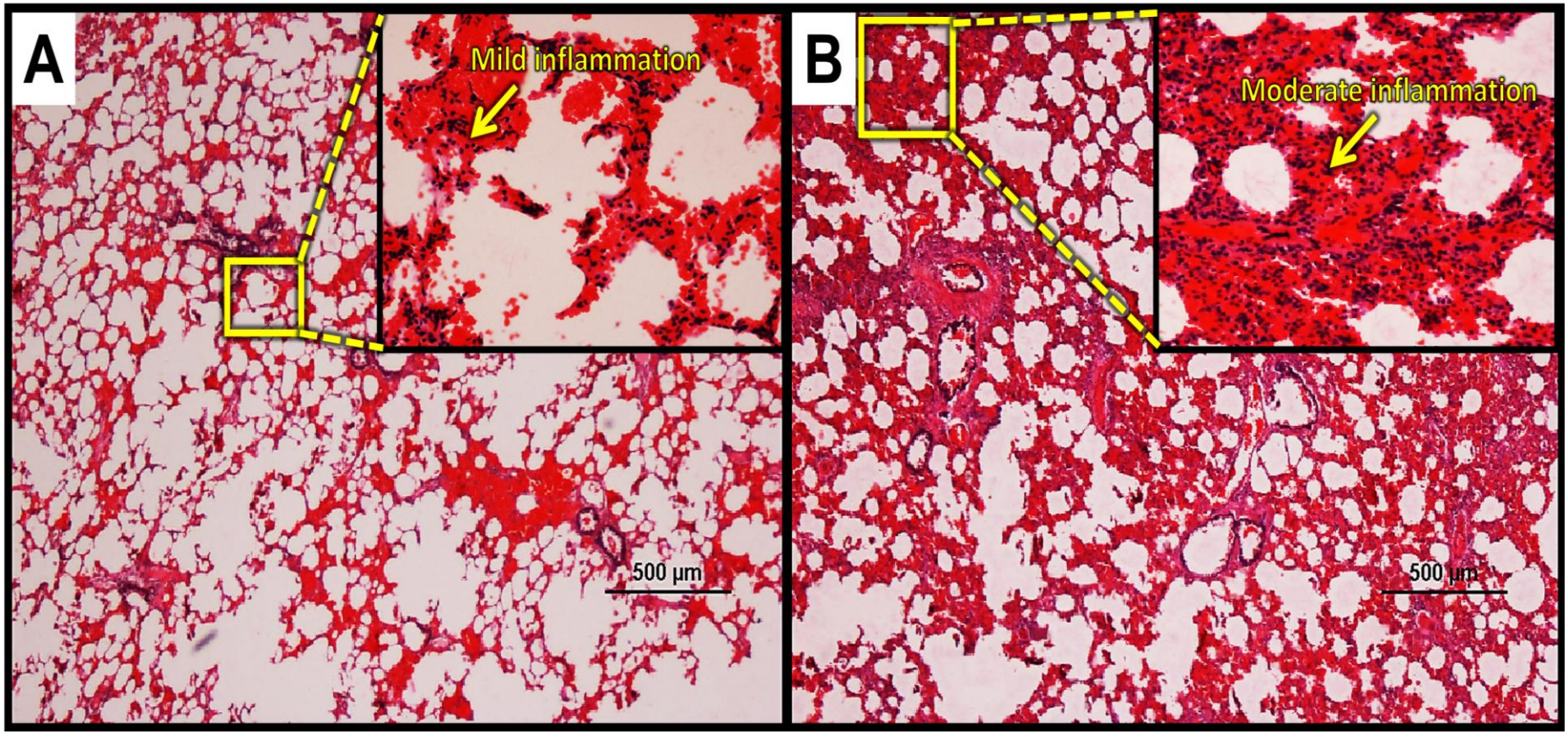

Figure 4.23 Histopathological scoring of lungs according to the severity of inflammation. (Scores II and III). **A,** Score II with mild focal inflammation of the lung (treatment group). **B,** Score III with moderate to severe focal inflammation of the lung (comparative control). Low magnification 4x. Arrows indicate the score of tissue and sections were stained with H&E.

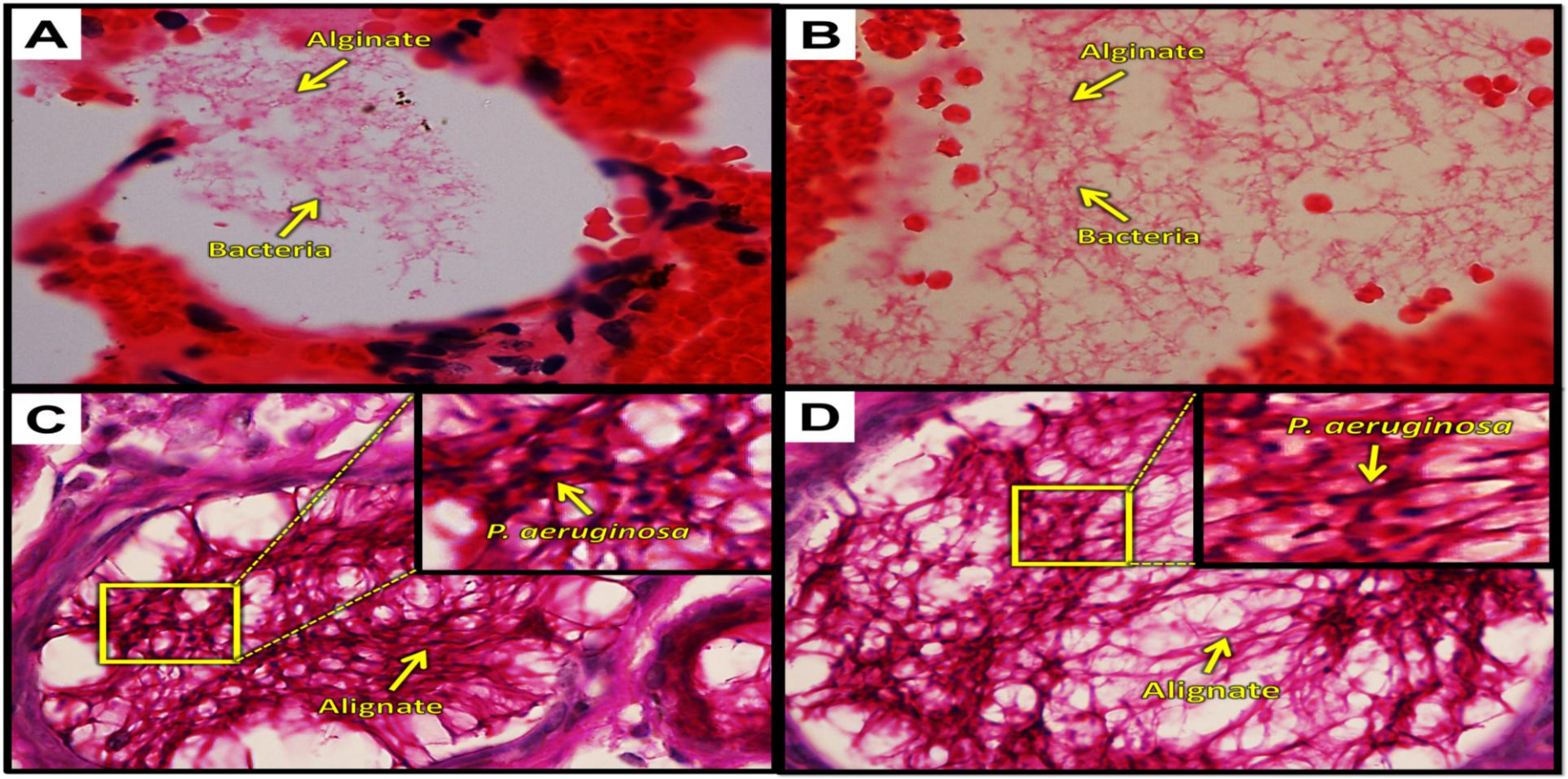

Figure 4.24 Microscopic evidence of *Pseudomonas aeruginosa* biofilms embedded with alginate solution inside the alveolar space of infected lungs. **A,** Biofilm occupying the alveolar space (Low magnification 40x). **B,** Biofilm occupying the alveolar space (high magnification 100x). **C and D,** Representative images of lung alveoli occupied with biofilms comprising of bacteria and alginate (high magnification 100x). Arrows indicate the biofilm structures, A and B tissue sections were stained with H&E stain whereas C and D sections were stained with PAS stain.

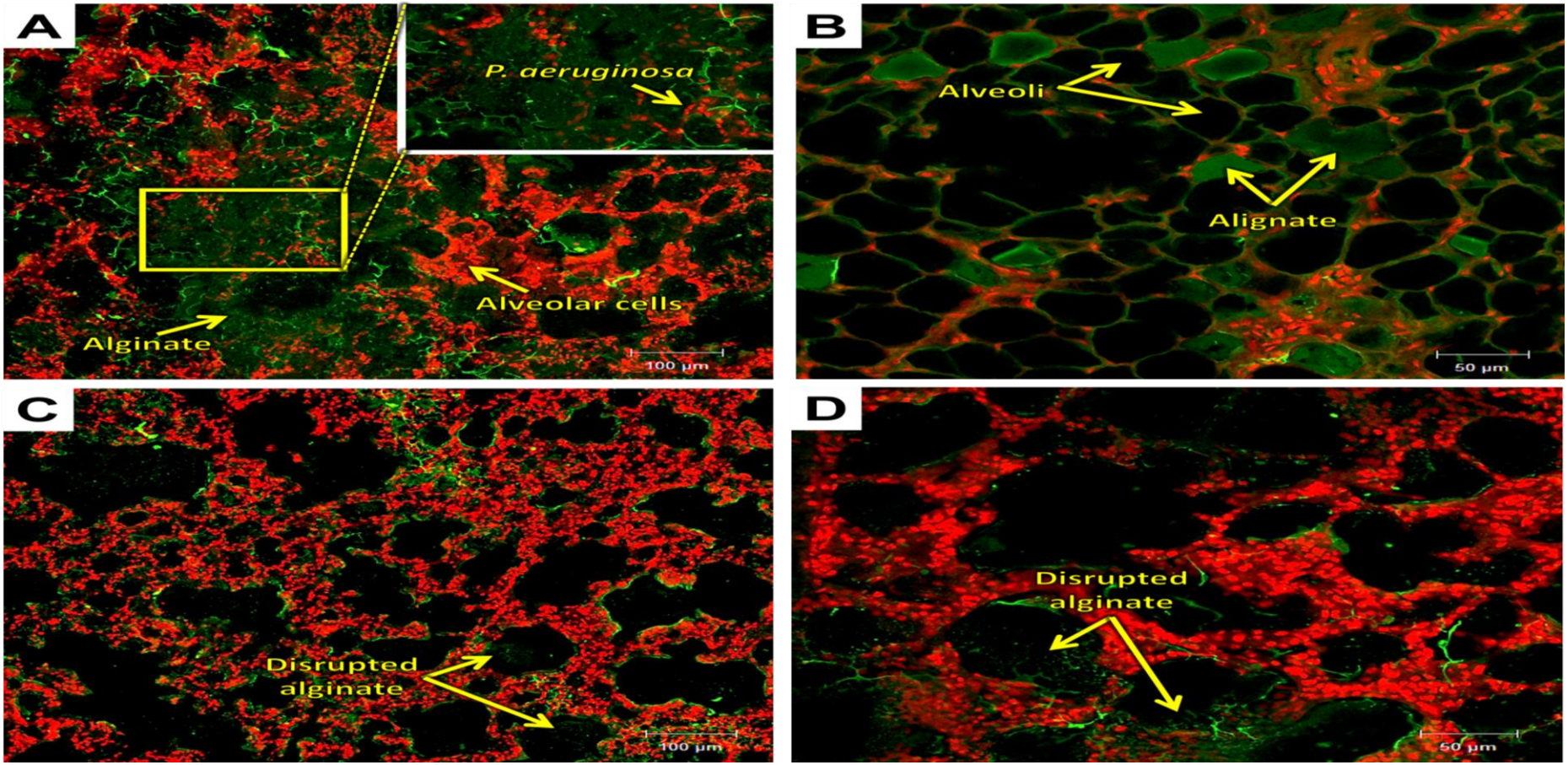

Figure 4.25 Microscopic evaluation of the lung pathology via CLSM according to the severity of inflammation. **A and B,** Representative images of positive (infection) control were dense layers of biofilm's alginate (green) are occupying the alveolar space and the *P. aeruginosa* and alveolar cells are shown (red). **C and D,** Representative images of treatment (139SI) group showing the therapeutic effects of 139SI filtrate in disrupting the biofilm's alginate leading to clear alveolar spaces and a minimized chronic infection. Arrows indicate the biofilm structures and tissue sections were stained with Propidium iodide & Concanavalin A.

4.11 Determination of 139SI Potential Compounds

The results of profiling the 139SI filtrate via HPLC revealed 32 fractions in which four exhibited antibiofilm activity (bioactive secondary metabolites) while the rest were non-active metabolites against biofilm formation. The HPLC chromatogram of 139SI filtrate with all its 32 fractions is shown in (Figure 4.26) whereas the four potential chemical compounds (FR4, FR5, FR8 and FR13) are shown in Figure 4.27 which were later further assessed and characterized.

4.12 Antibiofilm Activity of 139SI Potential Compounds

Among all the purified 32 fractions, only four showed significant inhibition of biofilm via MTP assay against Gram-negative isolates (Table 4.12) and Gram-positive isolates (Table 4.13). All the four fractions with their BIC exhibited antibiofilm activity when quantified spectrophotometrically. However, compound 5 (FR5) was the most active one with significant decreased in the adherence index compared to the rest of compounds.

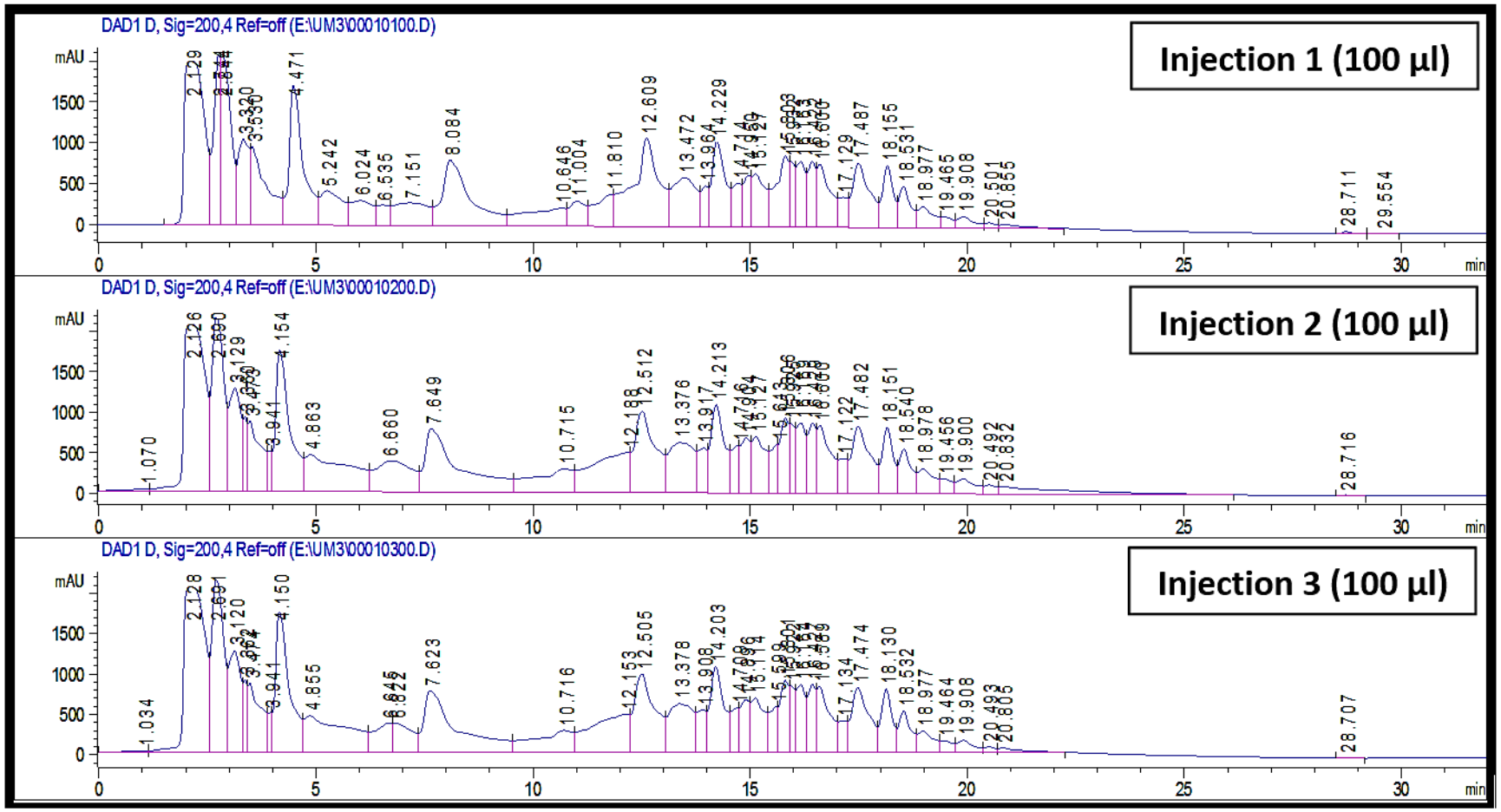

Figure 4.26 HPLC profiling chromatogram of 139SI filtrate showing all the obtained 32 compounds.

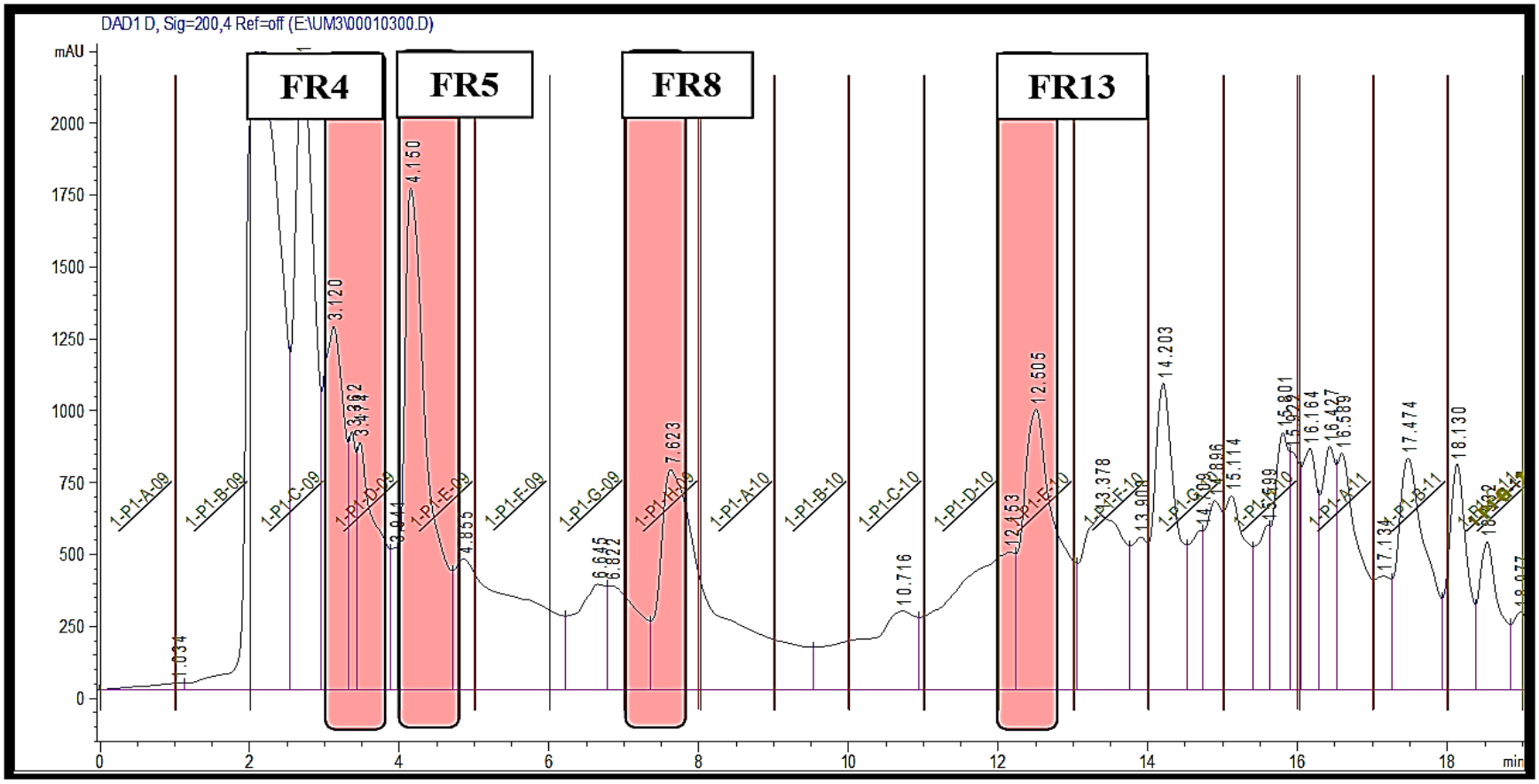

Figure 4.27 HPLC profiling chromatogram of 139SI filtrate showing the four potential compounds (FR4, FR5, FR8 and FR13).

Table 4.10.2 Antibiofilm activity of 139SI potential compounds against Gram-negative isolates. The most active compound (FR5) is highlighted.

139SI Potential Compound	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Citrobacter sp.*	Biofilm-forming reference strain *P. aeruginosa* (ATCC 27853)	Non-biofilm-forming reference strain *E. coli* (ATCC 25922)
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
FR 4	0.235 ± 0.005	0.245 ± 0.004	0.266 ± 0.004	0.194 ± 0.003	0.175 ± 0.004	0.225 ± 0.004	0.164 ± 0.004
FR 5*	0.192 ± 0.007	0.228 ± 0.009	0.245 ± 0.004	0.177 ± 0.005	0.165 ± 0.002	0.204 ± 0.003	0.147 ± 0.003
FR 13	0.255 ± 0.003	0.206 ± 0.004	0.257 ± 0.005	0.215 ± 0.004	0.182 ± 0.002	0.245 ± 0.003	0.155 ± 0.004
2(5H)-Furanone (Positive Control)	0.120 ± 0.004	0.092 ± 0.004	0.106 ± 0.004	0.116± 0.004	0.119 ± 0.002	0.124 ± 0.003	0.105 ± 0.004

OD = Optical Density, SD = Standard Deviation

Table 4.10.2 Antibiofilm activity of 139SI potential compounds against Gram-positive isolates. The most active compound (FR5) is highlighted.

139SI Potential Compound	*Staphylococcus aureus*	*Streptococcus agalactiae*	Group G Streptococci	*Streptococcus pyogenes*	*Streptococcus pneumoniae*	Biofilm-forming reference strain *S. aureus* (ATCC 25923)	Non-biofilm-forming reference strain *E. coli* (ATCC 25922)
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
FR 4	0.254 ± 0.004	0.205 ± 0.005	0.199 ± 0.010	0.191 ± 0.003	0.230 ± 0.004	0.246 ± 0.004	0.186 ± 0.004
FR 5 *	0.224 ± 0.004	0.166 ± 0.005	0.158 ± 0.002	0.153 ± 0.005	0.196 ± 0.004	0.188 ± 0.005	0.154 ± 0.003
FR 13	0.208 ± 0.004	0.205 ± 0.004	0.195 ± 0.004	0.216 ± 0.004	0.235 ± 0.004	0.254 ± 0.004	0.165 ± 0.004
2(5H)-Furanone (Positive Control)	0.119 ± 0.002	0.123 ± 0.003	0.116 ± 0.004	0.117 ± 0.001	0.121 ± 0.001	0.107 ± 0.004	0.116 ± 0.002

OD = Optical Density, SD = Standard Deviation

4.13 Identification of 139SI Potential Compounds

The compound identification was performed by UPLC–MS experiment in positive mode. A total number of six compounds were detected from the three active fractions obtained previously from HPLC in which they exhibited in vitro antibiofilm activity. Two compounds were detected from Fraction 4 (FR4), three compounds were detected from fraction 5 (FR5) and one compound was detected from fraction 13 (FR13). All detected compounds were identified on the basis of the UV spectra and MS fragmentation patterns in comparison with literature or by searching the dictionary of natural products on DVD, Version 20:2 (2011) (CRC Press, Taylor & Francis Group, London, UK). Table 4.14 shows the identification of all the peaks detected with their retention time, UV max, observed m/z and the m/z of fragment ions. Typical HPLC-TOF/MS peaks and UV diode array chromatograms of fractions 4, 5 and 13 are shown in (Figure 4.28, 4.30 and 4.32) respectively. Moreover, Mass Spectrum (TOF MS ES+), chemical structure and UV max spectra of the identified (peak No. 1) fractions 4, 5 and 13 are shown in (Figure 4.29, 4.31 and 4.33) respectively. The ssuggested elemental composition of the active compound FR5 using LC-MS in the positive mode is shown in (Figure 4.34).

In the negative mode: The chemical compound was with the synonym SF2415A2 with a molecular weight of 450.533 and a molecular formula of $C_{26}H_{30}N_2O_5$ described as a naphthoquinone related-antibiotic with an activity against Gram positive bacteria. However, in the positive mode: The first chemical compound was with synonym Leucine 2-(hydroxymethoxyphosphinyl)-2-methylhydrazide with a molecular weight of 253.237 and a molecular formula of $C_8H_{20}N_3O_4P$ described as an amino acid antibiotic with an activity against Gram positive and Gram negative bacteria. The second chemical compound was with the synonym 4-Hydroxy-5-(Hydroxymethyl)-3-(14-methylpentadecanoyl) tetronicacid-2(5H)-Furanone with a molecular weight of

368.512 and a molecular formula of $C_{21}H_{36}O_5$ described as a phospholipase A2 inhibitor. The third chemical compound was with synonym 6-(hydroxymethyl)-1-phenazinecarboxyamide with a molecular weight of 253.260 and a molecular formula of $C_{14}H_{11}N_3O_2$ described as an antibacterial agent. All detected compounds were identified on the basis of the UV spectra and MS fragmentation patterns in comparison with literature by searching the dictionary of natural products on DVD, Version 20:2 (2011) (CRC Press, Taylor & Francis Group, London, UK).

Table 4.10.24 Identified peaks of the 139SI filtrate filtrate detected with their retention time, UV max and m/z fragment ion.

Fraction	Peak	Retentions Time	Mode	Mass-to-charge ratio (m/z)	Molecular Formula
FR4	A	0.303	+	235.1196	$C_{11}H_{15}N_4O_2$
	B	0.410	+	235.1211	$H_{15}N_{10}O_5$
FR4	A	0.303	-	483.2881	$C_{28}H_{39}N_2O_5$
	B	0.399	-	444.2234	$C_{21}H_{34}N_1O_9$
	B	0.399	-	233.1024	$C_{11}H_{13}N_4O_2$
	B	0.399	-	483.2859	$C_{21}H_{34}N_1O_9$
	B	0.399	-	233.1023	$C_{10}H_{17}O_6$
	B	0.399	-	444.2241	$C_{21}H_{34}N_1O_9$
FR5	A	0.303	+	254.1622	$C_{11}H_{20}N_5O_2$
	B	0.736	+	369.1102	$C_{24}H_{17}O_4$
	B	0.736	+	210.0507	$C_8H_8N_3O_4$
FR5	A	0.318	-	237.1248	$C_{12}H_{17}N_2O_3$
	B	0.535	-	249.1337	$C_{12}H_{17}N_4O_2$
	C	0.715	-	351.1321	$C_{20}H_{19}N_2O_4$
	C	0.715	-	270.1468	$C_{17}H_{20}N_1O_2$
	D	1.016	-	355.1972	$C_{15}H_{31}O_9$
	D	1.016	-	499.2503	$C_{25}H_{39}O_{10}$
FR13	A	1.383	+	508.2532	$C_{26}H_{38}N_1O_9$
FR13	A	1.376	-	506.2310	$C_{21}H_{36}N_3O_{11}$

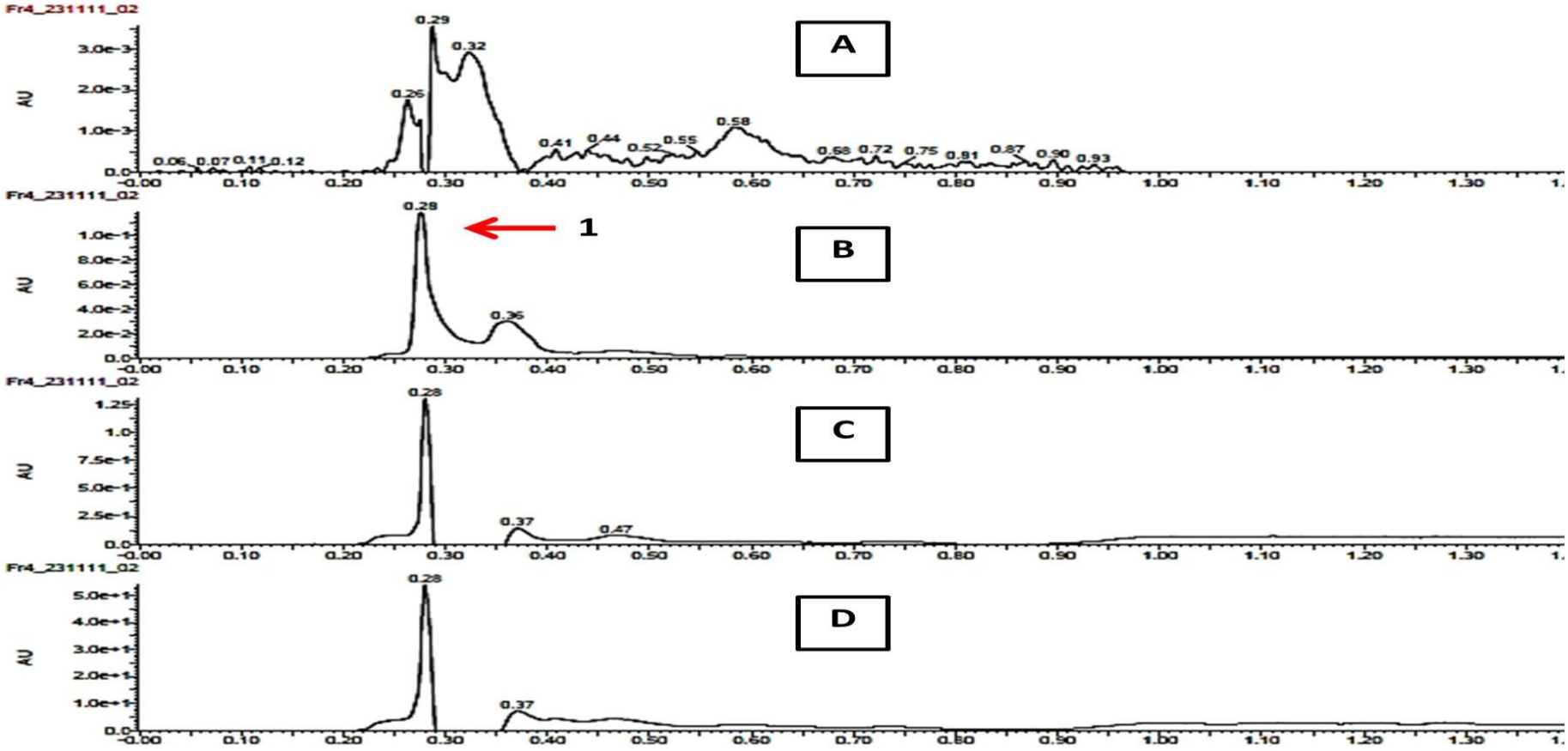

Figure 4.28 HPLC-TOF/MS and UV diode array chromatograms of 139SI fraction 4 (FR4) showing the peak in positive mode ionization. A, Diode array detection UV spectra at 280 nm. B, Diode array detection UV spectra at range between 190 and 800 nm.

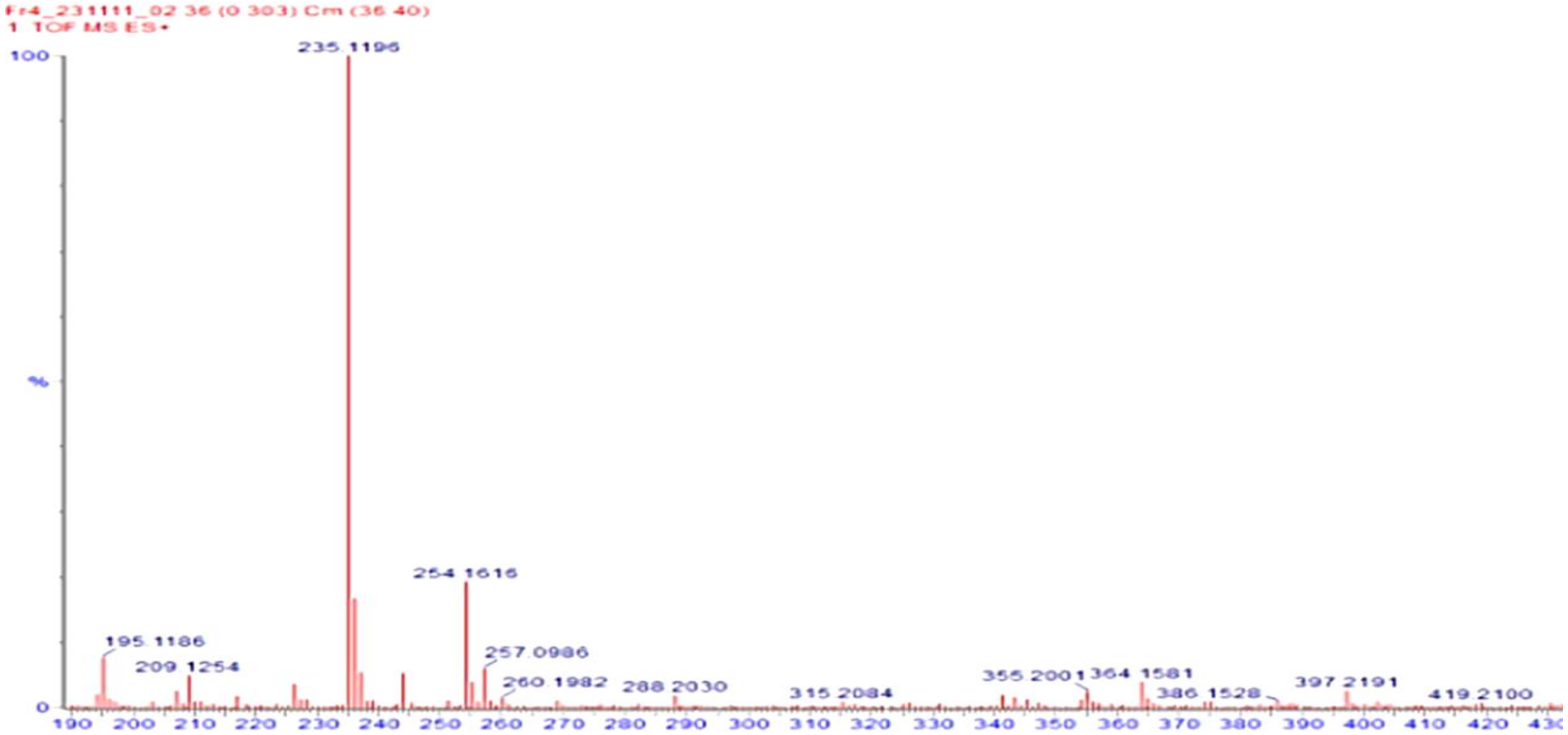

Figure 4.29 Mass Spectrum (TOF MS ES+) of peak No. 1 from fraction 4 (FR4) with its suggested elemental composition.

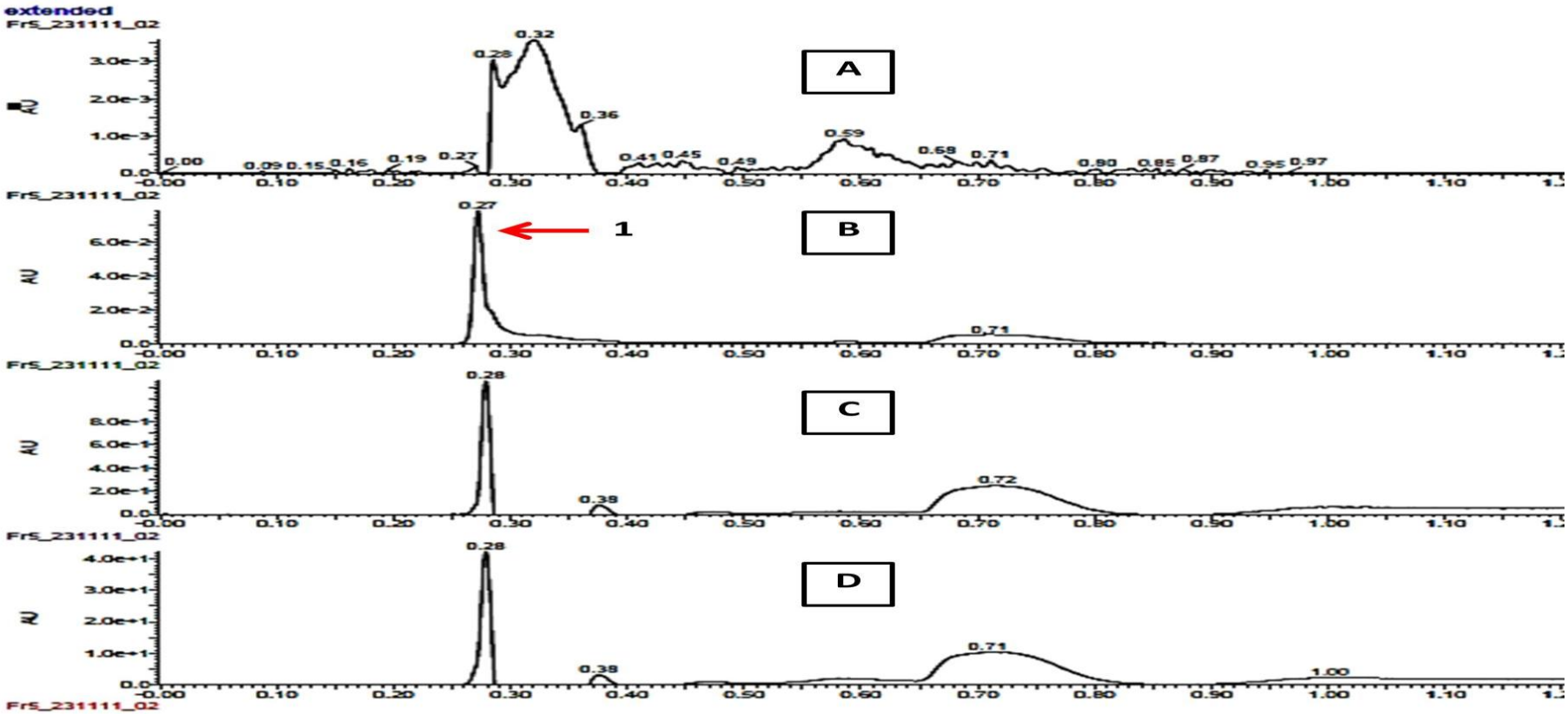

Figure 4.30 HPLC-TOF/MS and UV diode array chromatograms of 139SI fraction 5 (FR5) showing the peak in positive mode ionization. A, Diode array detection UV spectra at 280 nm. B, Diode array detection UV spectra at range between 190 and 800 nm.

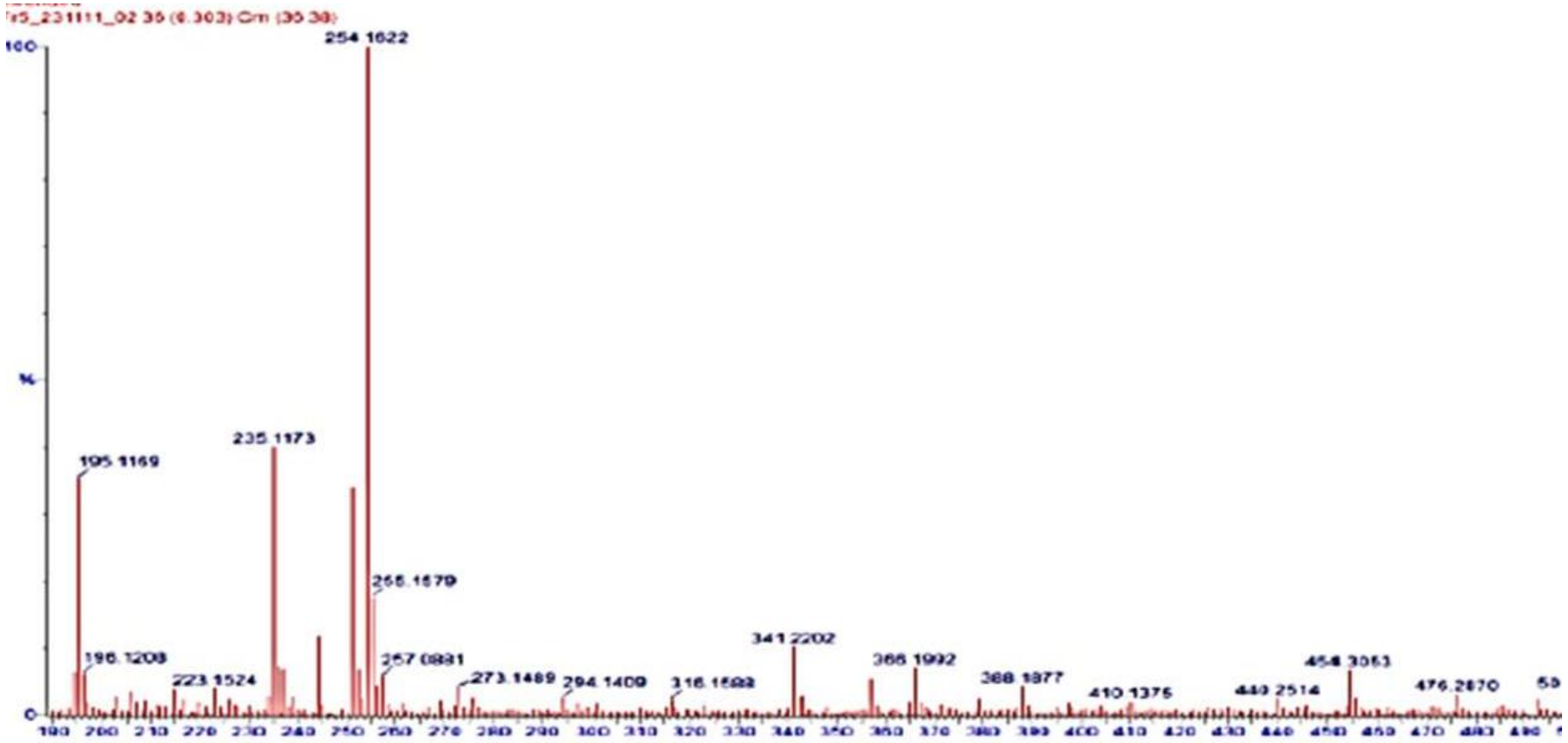

Figure 4.31 Mass Spectrum (TOF MS ES+) of peak No1 from fraction 5 (FR5) with its suggested elemental composition.

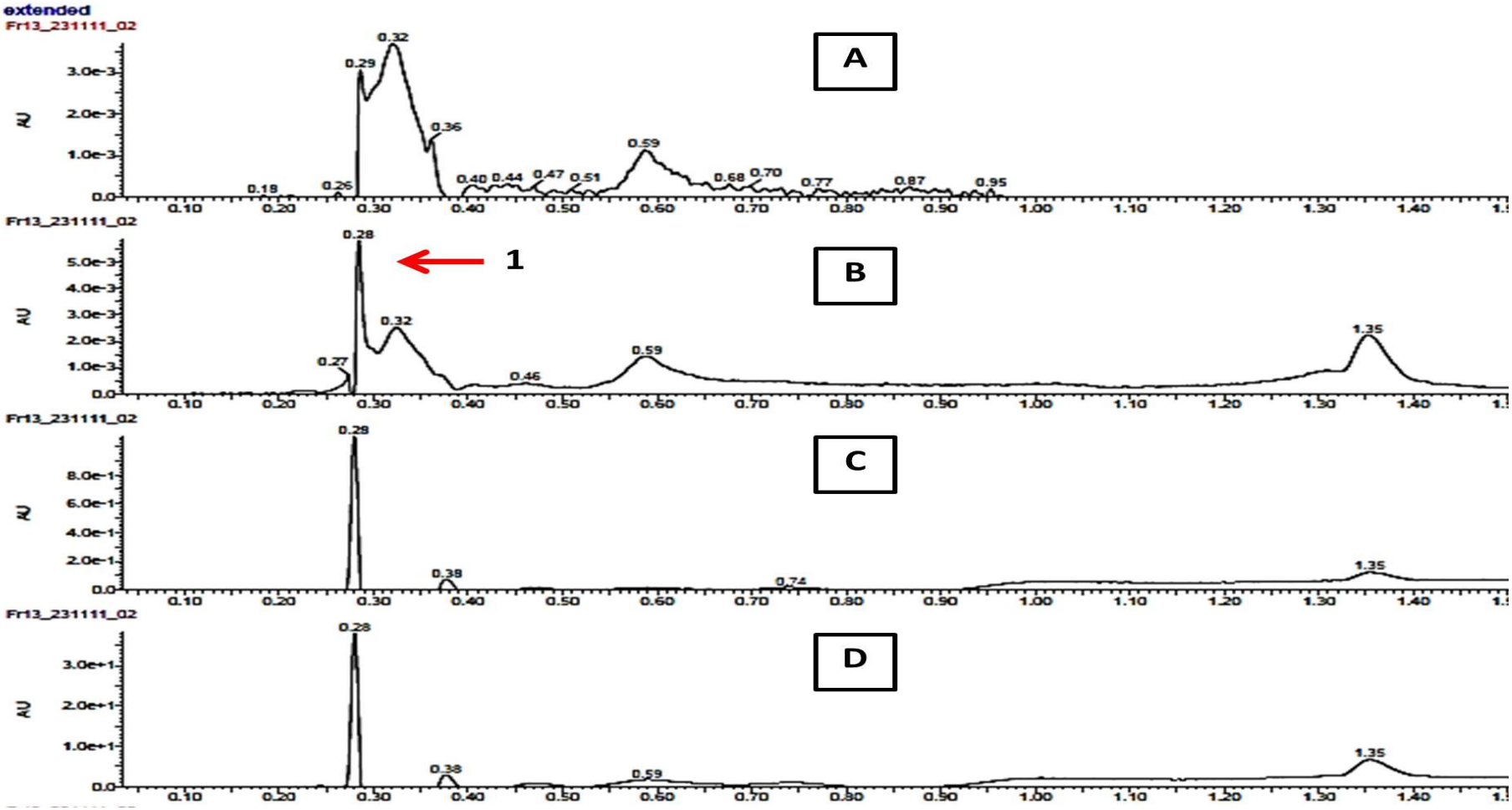

Figure 4.32 HPLC-TOF/MS and UV diode array chromatograms of 139SI fraction 13 (FR13) showing the peak in positive mode ionization. A, Diode array detection UV spectra at 280 nm. B, Diode array detection UV spectra at range between 190 and 800 nm.

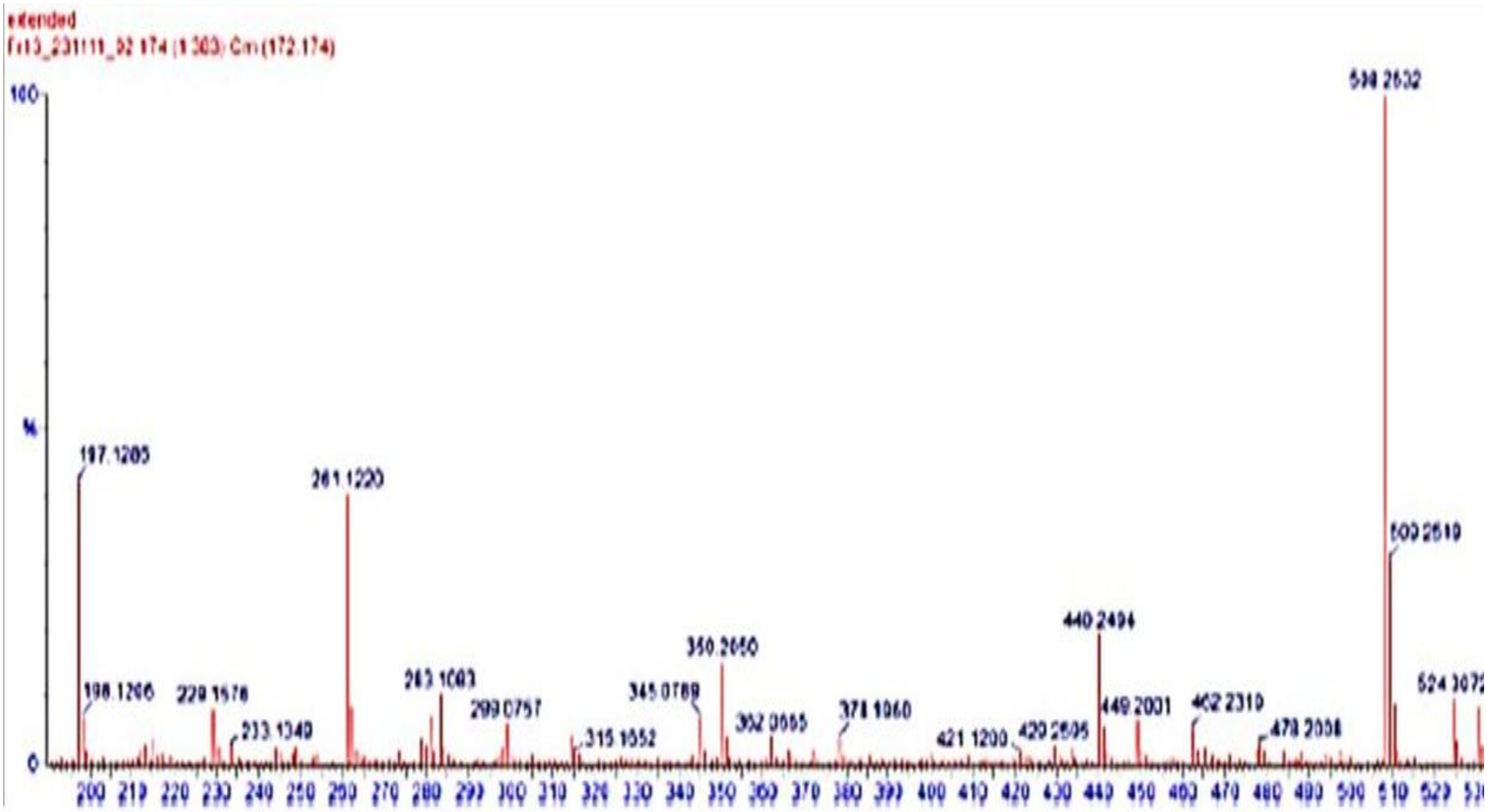

Figure 4.33 Mass Spectrum (TOF MS ES+) of peak No. 1 from fraction 13 (FR13) with its suggested elemental composition.

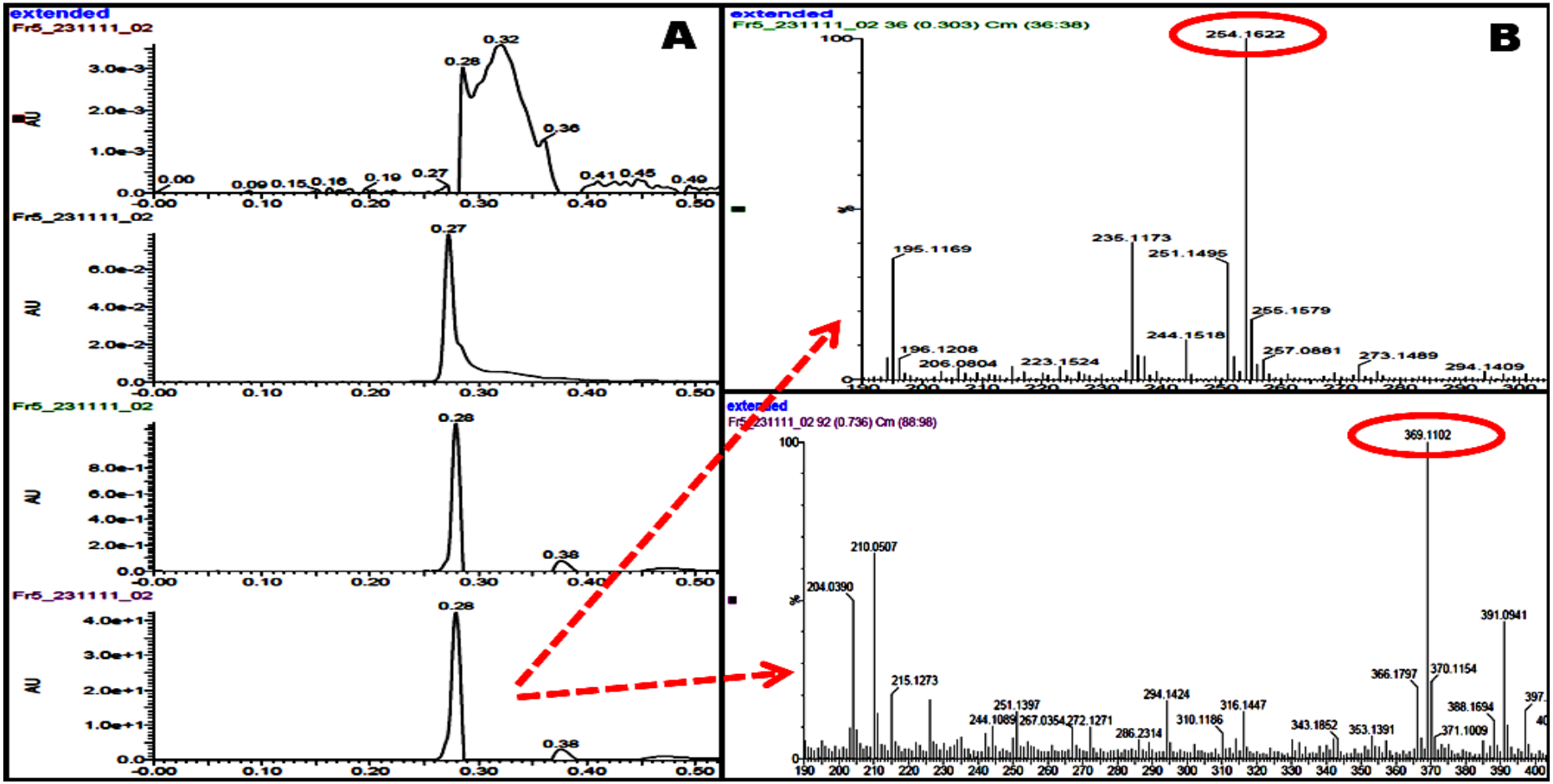

Figure 4.34 Suggested elemental composition of the active compound FR5 using LC-MS in the positive mode. **A,** Diode array detection UV spectra at 280 nm. **B,** Mass Spectrum (TOF MS ES+) of 2 peaks showing two chemical compounds, Leucine 2-(hydroxymethoxyphosphinyl)-2-methylhydrazide (MW = 253.237) ($C_8H_{20}N_3O_4P$). The second chemical compound is 4-Hydroxy-5-(Hydroxymethyl)-3-(14-methylpentadecanoyl) tetronic acid-2(5H)-Furanone (MW = 368.512) and formula ($C_{21}H_{36}O_5$).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Overview

Diseases of the ear, nose and throat (ENT) are one of the most frequent health problems especially in children that usually require surgical intervention. Among them, is tonsillitis which represents a real challenge due to its resistance to common therapies (Nixon and Bingham, 2006; Vlastarakos *et al.*, 2007). Despite the widespread use of antibiotics against tonsillar diseases, therapy is often insufficient and infections still occur and become chronic causing enlargement (hypertrophy) of the tonsils (Rehl *et al.*, 2007). Chronic tonsillar infections and hypertrophy are frequently caused by multiple and, sometimes, resistant bacteria. Many of these bacteria have the ability to form biofilms that are matrix-encased communities attached to a surface (Diaz *et al.*, 2011). One of the suitable surfaces that bacteria usually attach and form biofilms are the tonsils (Al-Mazrou and Al-Khattaf, 2008). In our study, we reported the presence of biofilms in 60% of selected patients and we established a clear relation between biofilms and some clinical symptoms of tonsillar diseases.

The failure of antibiotic treatments in tonsillitis might be due to the presence of biofilms that can be considered as an etiological factor for tonsillar diseases whether due to infection and/or obstruction. The knowledge about biofilms is crucial in explaining the chronic nature of bacterial infections including those associated with the ear, nose and throat (Vlastarakos *et al.*, 2007). In addition to detection of the presence of biofilms in the tonsils, we have isolated and identified the microorganisms from tonsillar specimens and assessed their ability to form biofilms in correlation with their antimicrobial susceptibility. Despite the fact that majority of our recovered tonsillar isolates were susceptible to a wide range of selected antibiotics, they exhibited a strong

ability to form biofilms. All of these results suggest that biofilms represent a new concept in ENT-related chronic infections, and are probably involved in their pathophysiology and in the antibiotic resistance which requires immediate attention to search for novel and effective therapeutic treatments.

Among the promising approaches to combat biofilm infections is the use of microbial natural products from microorganisms inhabiting natural environments. In our study, a taxonomically novel bacterial species that belongs to the genus *Paenibacillus* was isolated from agricultural soil and investigated for its ability to synthesize and secret bioactive metabolites that can disrupt the biofilm formation of clinically important pathogens. Due to the remarkable haemolytic ability of our novel bacterial isolate, it was designated the name *Paenibacillus haemolyticus* strain 139SI. We have prepared a culture filtrate from this strain and tested it against a wide range of bacterial isolates previously recovered from tonsils. The culture filtrate of strain 139SI was tested for its *in vitro* antibiofilm activity, gross and histopathological toxicity, and therapeutic effects against biofilm-forming pathogens. Our findings suggest that the 139SI culture filtrate exhibited potent antibiofilm activity with no signs of toxicity and had significantly reduced a chronic biofilm infection in the lungs of experimental animals.

Profiling of the crude culture filtrate of strain 139SI via high performance liquid chromatography (HPLC) has yielded a total number of 32 compounds (fractions). Each fraction was then tested for its *in vitro* antibiofilm activity via MTP assay and results revealed three potential fractions designated FR4, FR5 and FR13, These fractions were selected based on reducing bacterial adherence on the surface of microtitter plate and thus inhibiting the formation of biofilm. Each fraction with their respective concentrations were tested again to determine their *in vitro* antibiofilm activity. Among the three fractions, FR5 was selected as the most active fraction against Gram-positive and Gram-negative pathogens and it was further characterized to identify its molecular

weight, chemical formula and chemical structure via liquid chromatography-mass spectrometry (LC-MS). Three potential chemical compounds were identified from FR5; where the first compound was Leucine 2-(hydroxymethoxyphosphinyl)-2-methylhydrazide (MW = 253.237) and ($C_8H_{20}N_3O_4P$) described as an amino acid antibiotic with an activity against Gram-positive and Gram-negative bacteria. The second compound was 4-Hydroxy-5-(Hydroxymethyl)-3-(14-methylpentadecanoyl) tetronicacid-2(5H)-Furanone (MW = 368.512) and ($C_{21}H_{36}O_{5)}$ described as a phospholipase A_2 inhibitor. The third compound was 6-(hydroxymethyl)-1-phenazinecarboxyamide (MW = 253.260) ($C_{14}H_{11}N_3O_{2)}$ described as an antibacterial agent.

The FR5 fraction (compound) is readily soluble in water and exhibited antibiofilm activities against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pneumonia)* and Gram-negative bacteria (*Haemophilus influenzae* and *Pseudomonas aeruginosa).* However, other characteristics like mechanism of action and stability properties need to be elucidated in future work with the hope for therapeutic applications in medicine especially against ENT-biofilm associated infections.

5.2 Pathology of Tonsillar Diseases

The gross pathology of our tonsillar biopsy specimens showed that the highest rate of tonsillar grading was found among grade III (3+) with 39 (55.71%) patients followed by grade II (2+) with 20 (28.57%) patients then grade I (1+) with 6 (8.57%) patients and grade IV (4+) with 5 (7.15%) patients. This was similar to the finding made by Dell'Aringa *et al.* where they reported the highest grade III (3+) with 160 (64%) patients followed by grade II (2+) with 45 (18%) patients, grade IV (4+) with 26 (10.4%) patients and grade I (1+) with 9 (3.6%) patients (Dell'Aringa *et al.*, 2005).

There was no malignant neoplasia in all tonsillar specimens, this could be due to the low percentage of patients under the age of 18 years submitted to surgery, resulting in low incidence of these lesions. This was consistent with Dell'Aringa *et al.* where they reported the absence of malignancy from their tonsillar specimens (Dell'Aringa *et al.*, 2005).

In our study we haven't evaluated the influence of tonsil size on obstructive sleep apnea patients and the influence of oropharyngeal anatomy, body mass index and age on actual tonsil volume. This was due to the fact we emphasized on the microbiology and histopathology aspects of tonsillar diseases rather than physiological and anatomic aspects. Therefore, no correlation between oropharyngeal examination and the volume and size of palatine tonsils of snoring patients was assessed. However, a prospective study by Cahali *et al.* where they studied 130 patients with obstructive sleep apnea or primary snoring who underwent pharyngeal surgery with intraoperative measurement of tonsil volume demonstrated a strong correlation between clinical tonsil grade and the objective volume of tonsils in snoring adult patients, and this correlation existed regardless of the presence or severity of obstructive sleep apnea (Cahali *et al.*, 2011).

According to the histopathological examination in our study, only 11 (15.71%) patients presented infections by *Actinomyces* sp. colonies in their tonsils leading to an enlargement of tonsils (hypertrophy) and an inflammatory lesion of the tonsillar crypts. This was consistent with the report by Sánchez *et al.* where they observed the presence of Actinomycosis in 8.5% of the patients with obstructive tonsillar hypertrophy and recurrent tonsillitis (Pransky *et al.*, 1991). However, it was in contrast with the results obtained by Dell'Aringa *et al.* (2005) where they reported only 2 patients (0.8%) with infections by *Actinomyces* sp. that led to tonsil hypertrophy (Dell'Aringa *et al.*, 2005)

Despite the presence of *Actinomyces* in our examined tonsillar tissue, the rate of these infections among clinical cases was considered low suggesting no relation between the presence of *Actinomyces* might cause hypertrophy of palatine and pharyngeal tonsils in these patients or, as *Actinomyces* is a common agent in the tonsillar tissue, it may not be routinely analyzed in the pathological specimens.

5.3 Microbiology of Tonsillar Diseases

Swabbing of tonsillar surface represents the routine method for bacteriological investigation of the tonsils. In our results, we have not been able to demonstrate a significant quantitative differences between surface (swab) and core (biopsy) cultures in regard to the recovery rate and type of aerobic bacteria. In fact, the microorganisms isolated from the tonsillar core could be also isolated from the surface. Therefore, the surface tonsillar swabs seem quite accurate in identifying the potential pathogens in chronic and recurrent tonsillitis as well as obstructive sleep apnea. This was similar to the investigation made by Alamadori *et al.* (1988) for which they have reported no significance qualitative difference between tonsillar surface and core cultures. However, it was in contrast with previous studies where they have demonstrated that the isolated microorganisms from tonsillar surface may not always represent the real cause of recurrent tonsillitis (Brook *et al.*, 1980; Rosen *et al.*, 1977). A study by Kurien *et al.* illustrated the problem of using the results of surface culture to determine the microorganisms responsible for tonsillar infections which in turn may lead to poor response to medical therapy in chronic and recurrent tonsillitis (Kurien *et al.*, 2000). Another study by Rosen *et al.* also reported that in 48% of the bacteria isolated from their swab cultures were different from those isolated from deep tissue culture (Rosen *et al.*, 1977). In addition, Brodsky *et al.* reported that the tonsil core bacteria with the

highest bacterial concentrations were more likely to be present on the tonsillar surface, and the greater the bacterial concentration, the more likely the bacteria were to be found in most if not all areas of the tonsil core (Brodsky *et al.*, 1991).

The only microorganism that was found to have significant difference between the surface and core distribution was *Haemophilus influenzae* for which 10 isolates where recovered from the core whereas 21 were recovered from the surface. This was similar to the study by Gul *et al.* which reported a significant difference between surface and deep tissue cultures, especially for *H. influenzae* and *S. aureus* isolates despite being common in core cultures, *H. influenzae* was rarely present on surface cultures (Gul *et al.*, 2007) indicating that the surface cultures commonly show commensal flora whereas the tonsil core cultures show pathogenic microorganisms. Despite the contrast with previous studies on the role of tonsillar surface to precisely reflect the type of microorganisms, we believe that the use of superficial swabs can still be reliable to recognize the presence of possible pathogens especially for patients who are not willing to undergo surgical management in spite of not responding to routine medical treatment.

Although there was no assessment of the role of anaerobic bacteria, viruses and fungi among our clinical specimens due to technical difficulties in collecting, transporting and culturing them, an accurate microbiological investigation towards isolating such microorganisms seems necessary in order to assess an adequate antimicrobial therapy prior to the occurrence of irreversible changes in the tonsillar flora that might to lead to a persistent infection.

Our assessment of the microbiology of tonsillar specimens showed that *Staphylococcus aureus* was the most common microorganism among Gram-positive isolates and *Haemophilus influenzae* the most common among Gram-negative isolates indicating that they might be an etiological factor for tonsillitis. This was similar to the findings by Forbes *et al.* (2002) and Kielmovitch *et al.* (1989) which showed

Staphylococcus aureus and *Haemophilus influenzae* as the main causes of tonsillitis. Similarly studies by Gunnarsson and coworkers (2001) and Le *et al.* (2007) where they reported *Haemophilus influenzae* and *Staphylococcus aureus* as pathogens that play a role in upper respiratory tract infections along with group C and G β-hemolytic streptococci. However, the low number of recovered *Streptococcus pneumoniae* and GABHS isolates from both infected and hypertrophied tonsils indicates a less likely role in the development of tonsillitis. This was in contrast to a similar study by Kielmovitch *et al.* (1989) where the authors reported GABHS, *Streptococcus pneumoniae*, *Corynebacterium diphtheriae*, and *Neisseria gonorrhoeae* as the main causes of tonsillitis.

In recurrent tonsillitis, the goal of the treatment is to eradicate the bacteria that cause infection. Inappropriate antibiotic therapy against pathogens in deep tissue or inadequate antibiotic levels in the tonsillar tissue leads to the continuation of the infection and the re-inoculation of the surface (Surow *et al.*, 1989). Because the tonsillar surface is coated with oropharyngeal secretions, it generally shows normal flora of the oropharynx such as α-hemolytic and non-hemolytic streptococci, coagulase negative staphylococci, *Neisseria*, *Flavescens*, *Corynebacterium*, *Actinomyces*, *Leptotrichiae*, and *Fusobacterium* species (Surow *et al.*, 1989).

Previous investigations showed that hypertrophied tonsils have increased numbers of pathogenic bacteria compared with non-hypertrophied ones (Kuhn *et al.*, 1995) However, in our study when comparing the microbial flora among infected and non-infected tonsillar cases there was no significant difference in the number of recovered bacterial isolates. This was similar findings made by Stewart and Costerton where the same microbial flora was found among the infected and hypertrophied tonsils (Stewart and Costerton, 2001) suggesting that hypertrophied tonsils even without a history of infection cannot be considered as control samples which added a limitation to

our study due to difficulties in obtaining tonsillar specimens from age-matched individuals who never had infection or obstruction in their upper airways.

In the case of a tonsillar infection, bacteria that inhabit the crypts spread into the tonsil and leave their toxins and other products in it, eventually leading to polymorphonuclear leukocyte infiltration, necrosis and surface ulceration in tonsils. Consequently, after an acute infection, bacteria may invade the tonsillar core (Brodsky *et al.*, 1988; Gross and Harrison, 2000; Uppal and Bais, 1989). However, the mechanism of activating such infection in recurrent tonsillitis is still poorly understood (Loganathan *et al.*, 2006). Therefore, knowing the bacteriology of tonsils does not help to treat the disease, however it helps develop an understanding of whether the bacteria play a role in re-activating recurrent infections by forming a biofilm community.

5.4 Antimicrobial Susceptibility of Tonsillar Diseases

Selective reporting of antimicrobial agents should help improve the clinical relevance of test reports and may assist to minimize the selection of multi-resistant nosocomial strains by overuse of broad-spectrum agents (Clinical and Laboratory Standards Institute, CLSI, 2009a). The Clinical Diagnostic Laboratory at UMMC in consultation with the infectious disease practitioners have decided which agents to report routinely and which might be reported only selectively. Therefore, based on their decision we have chosen the agents to be tested on each of our clinical isolates.

Antimicrobial dosage regimens often vary widely among practitioners and institutions. Isolates that are initially susceptible may become intermediate or resistant after initiation of therapy (Clinical and Laboratory Standards Institute, CLSI 2010). Our results from disk diffusion tests showed that there is a high percentage of sensitivity among majority of tonsillar isolates against the selected antibiotics. These findings are

similar to the results by Sadoh *et al.* where the authors reported 100% sensitivity to cefuroxine, azithromycin and ceftazidime among *S. aureus* and Beta Haemolytic Streptococci (Sadoh *et al.*, 2008). In our study, ampicillin showed more resistance against *P. aeruginosa* and *H. influenzae* isolates. Although the reason for this resistance is not clear, it may be related to the inappropriate consumption of the ampicillin that will eventually develop resistance. This was consistent with findings by Sadoh *et al.* (2008) when they tested their throat swabs bacterial isolates against selected antibiotics.

We have detected a noticeable percentage of resistance to trimethoprim-sulfamethoxazole (co-trimoxazole). These include 21.43% resistance by GABHS, 68.60% resistance by *H. influenzae* and 67.75% by *H. parainfluenzae*. This resistance was consistent with findings by Sadoh *et al.* (2008) when they tested the throat swabs samples against ampicillin and co-trimoxazole.

Although our antimicrobial susceptibility data cannot estimate the current status of antibacterial resistance in Malaysia, it highlights a number of potentially important issues regarding the antibacterial susceptibility and epidemiology of the key respiratory tract pathogens such as *S. aureus*, *H. influenza* and GABHS (Alasil *et al.*, 2011). Despite our results that showed high antimicrobial susceptibility against majority of the selected antibiotics, there was a noticeable resistance to fusidic acid by the pathogen *S. aureus*. This finding was consistent with the study made by Brown and Thomas (2002) where they reported a 10.6% increased rate of resistance to fusidic acid among methicillin-susceptible *S. aureus* (MSSA) isolates make it a less potential drug of choice for patients with chronic and recurrent tonsillitis. This was similar to another study by Norazah *et al.* (2002) reporting an increased resistant to fusidic acid between 3 to 5% among Malaysian hospitals.

The fact that 12 (2.58%) of our tonsillar *Haemophilus influenzae* isolates were found to be β-Lactamase Negative Ampicillin-Resistant (BLNAR) is a big concern.

Resistance of *H. influenzae* to ampicillin has been increasing steadily since its emergence in the 1970s. Until recently, β-lactamase production has been the primary mechanism of ampicillin resistance among *H. influenzae*. However, the prevalence of β-Lactamase-Nonproducing Ampicillin-Resistant (BLNAR) strains of *H. influenzae* now also appears to be increasing (Clinical and Laboratory Standards Institute, CLSI, 2003). This is of clinical significance, since BLNAR *H. influenzae* are typically co-resistant to other commonly prescribed β-lactams, including Augmentin (i.e. amoxicillin-clavulanate) and ampicillin-sulbactam, in addition to most cephalosporins (Clinical and Laboratory Standards Institute, CLSI, 2003).

Since ESKAPE pathogens are responsible for a high percentage of nosocomial infections, interestingly we have found that the ESKAPE pathogens recovered from our tonsillar specimens showed high susceptibility against selected antibiotics except for *Pseudomonas aeruginosa* with 22.22% resistance (Appendix 10L) which was in contrast with the resistant ESKAPE pathogens previously reported by Rice (2010).

The difficulty of treating biofilm infections with antibiotics is a major clinical problem (Ciofu and Tolker-Nielsen, 2011). Bacterial interference has been shown to exist between Alpha-Haemolytic Streptococci (AHS) and Beta-Haemolytic Streptococci and between Gram Negative Bacilli (GNB) and AHS recovered from tonsillar regions (Holm and Grahn, 1983). Study by Yoshioka *et al*. (1982) they demonstrated that oral ampicillin, cephalothin, tetracycline and chloramphenicol have been shown to suppress the AHS and promote the emergence of GNB. The lack of interference strains may explain the increased susceptibility of certain individuals to β-haemolytic streptococci. Preservation of the normal flora has more advantages than their re-establishment by antibiotics (Brook, 2007). Since the administration of antimicrobial agents can affect the composition of the nasopharyngeal bacterial flora, including the reduction of interfering

organisms, a proper use of antimicrobial agents is important in the preservation of the normal interfering flora (Brook and Gober, 2005).

The lack of a rapid and reproducible assays that provide accessible assessment of activity of antibiotics with efficacy against bacterial biofilms has become a problem in selecting alternative antibiotic regimes (Ceri *et al.*, 1999; Olson *et al.*, 2002). Therefore, antimicrobial susceptibility of bacteria in a biofilm should be assessed especially when dealing with chronic infections caused by microorganisms for which their MICs have not provided clinically relevant information. This will assist clinicians in the selection of more powerful antibiotics for their activity and efficacy against biofilms.

5.5 Evidence of Biofilms in the Tonsils

Tonsillar and adenotonsillar diseases such as adenotonsillar hypertrophy causing obstructive sleep apnea syndrome (OSA) and/or chronic adenotonsillitis present a major problem in children and are the most common indications for tonsillectomy and/or T&A (Al-Mazrou and Al-Khattaf, 2008). Many of these infections are thought to be caused by resistant bacteria that have the ability to form biofilms (Don *et al.*, 2005). Therefore, proper detection of such biofilms is of great importance for better management of tonsillar and adenotonsillar diseases.

Our results showed the presence of attached bacteria in the form of biofilms on the surface of the palatine tonsils in 42 (60%) of selected patients via SEM and CLSM. The distribution of biofilms was 30 out of 49 patients with recurrent tonsillitis, 5 out of 9 patients with chronic tonsillitis and 7 out of 12 patients with obstructive sleep apnea. This was similar to results published by Kania *et al.* (2007) who detected the presence of bacterial biofilms on human tonsillar mucosal tissue in (70.8%) patients with

tonsillitis via CLSM and SEM. Another similar study by Chole and Faddis (2003) demonstrated the presence of bacterial biofilms as acellular deposits among the crypts of 11 of 15 infected tonsils and 3 of 4 hypertrophied tonsils.

Recovery of clinical isolates from infected and hypertrophied tonsils is important. However, the detection of biofilm formation by these isolates is thought to be of great impact to overcome therapeutic failures. Our study showed the presence of bacterial biofilms in infected and hypertrophied tonsils from patients with a history of infection and obstruction which suggest the role of biofilms in the pathogenesis of these clinical conditions and raise the possibility that bacterial biofilms are part of tonsillar microbial flora among clinically diseased tonsils which may explain the recalcitrant nature of some cases of recurrent tonsillitis (Chole and Faddis, 2003).

Moreover, the fact that biofilms were observed in 7 out of 12 of the hypertrophic tonsils confirm the hypothesis that not only tonsillar infections but tonsillar hypertrophy is one of the important symptoms associated with the presence of biofilms. This was similar to the findings described by Chole and Faddis (2003) who reported that bacteria in biofilms are sequestered from host defenses and antibiotics which may explain the recalcitrant nature of some cases of chronic and recurrent tonsillitis.

Based on the histological characteristics of tonsillar specimens, the number of lymphatic follicles was related to the presence of biofilms in infected more than non-infected tonsils similar to the results obtained previously by Diaz *et al.* which might be related to the fact that most of the patients were diagnosed with infection-related symptoms rather than obstruction (Diaz *et al.*, 2011). Despite the low prevalence of symptoms like apnea and nasal obstruction in comparison with tonsillar and adenoid hypertrophy among our selected patients, a direct correlation between apnea and nasal obstruction was found with the presence of biofilms in 7 out of 12 hypertrophied tonsils. This finding is consistent with previous investigations (Diaz *et al.*, 2011).

Failure of patients with tonsillitis to respond to antimicrobial therapy leaves no choice but tonsillectomy. However, despite the role of tonsillectomy in relieving the symptoms of tonsillar diseases, the more likely explanation for its effectiveness is the elimination of a possible microbial biofilm infection. This is suggested by previous observations of bacterial resistance within a biofilm which confirms the hypothesis that chronic and recurrent tonsillitis are biofilm-related (Richardson *et al.*, 1980; Stewart and Costerton, 2001).

The fundamental knowledge and rapid detection of biofilms can lead to a better therapeutic management of tonsillar diseases to become more effective and less invasive (Suh *et al.*, 2010). However, the demonstration of biofilms in human tissues is technically challenging and has so far relied mostly on the use of scanning electron microscopy and transmission electron microscopy (Kania *et al.*, 2007). During sample preparation for SEM, the biofilm structure can be damaged which will add a limitation to clearly visualize biofilms (Akiyama *et al.*, 2003). Despite the fact that image acquisition of biofilms obtained from SEM were correlated with the image acquisition obtained from CLSM, presence of biofilms via SEM in some of our samples was hampered by cell clots and mucus on the surface of the tissue leading to mask the bacteria from being properly visualized. This finding was similar to a study by Kania *et al.* where they reported that the images obtained by SEM are largely affected by the specimen's dehydration (Kania *et al.*, 2007) which can lead according to their report for false positive biofilm-like artifacts. Therefore, we conclude that detecting the bacteria and its glycocalyx is crucial for a fundamental understanding of the presence of biofilms in clinical specimens which can be largely supported through the use of CLSM.

Our results indicates that the prevalence of bacterial biofilms among the 5 clinical cases of Streptococcal Tonsillitis (ST) was 100% while among the 30 clinical cases of Non-Streptococcal Tonsillitis (NST) was 53% indicating a role of both

GABHS and non-GABHS isolates in the pathogenesis of biofilm-associated infection and obstruction tonsillar diseases among both children and adults. This was similar to findings made by Diaz *et al.* (2011) during a study which investigated the correlation between chronic inflammation of the tonsils, clinical features and the presence of biofilms in the crypts in 36 patients undergoing tonsillectomy for obstructive sleep apnea and recurrent upper airway pathology (Diaz *et al.*, 2011). Their results showed that symptoms like harsh raucous sound, tonsillar and adenoids hypertrophy, apnea, and cervical adenopathies are clearly related to the presence of biofilm in tonsils allowing them to conclude that biofilms are involved in the pathogenesis of tonsils and adenoids hypertrophy. Therefore, the prevention of biofilms formation should be focused in the early stages, attempting to restrain bacterial attachment to the respiratory mucosa.

The absence in reporting a biofilm infection via routine clinical pathology examination is maybe due to the conventional techniques that are still being used in most histopathology labs such as light microscope coupled with routine staining such as H&E which might hamper the ability to precisely detect a source of infection as important as a biofilm. Therefore, for a fundamental understanding and proper examination of bacterial biofilms in tissue sections, detection must include both the bacteria and its glycocalyx matrix (Kania *et al.*, 2007). This can be achieved by using Confocal Laser Scanning Microscopy (CLSM) (Oliveira and Cunha Mde, 2010) coupled with a double staining technique which will allow to visualize microbial biofilms and observe their 3-dimensional structure in a way that routine histology methods cannot, thus providing a more realistic image of the biofilm *in situ*. This observation was similar to previous reports (Kania *et al.*, 2007; Oliveira and Cunha Mde, 2010; Rumbaugh and Carty, 2011) where they emphasized on the role using novel visualization approaches such as CLSM to examine biofilms in single sections of human mucosal tissue such as the tonsils.

The use of confocal laser scanning microscopy allows a better examination of biofilms, and this is mainly achieved by the use of certain fluorescent dyes such as Concanavalin A that binds to mannose residues specific to the bacteria's glycocalyx (Zur *et al.*, 2004). Moreover, the use of various software functions within the CLSM have a big impact on analyzing important parameters within the biofilm such as the degree of co-localization of certain labeled structures (Kania *et al.*, 2007). However, despite the importance of using CLSM to visualize biofilms, it has the limitation of not identifying the type of microorganisms causing that biofilm in addition of being costly and time-consuming in terms of sample processing and analysis. This finding was similar to what was reported previously (Kania *et al.*, 2007).

Our results demonstrated that clinical symptoms like snoring, apnea, nasal obstruction, tonsillar and adenoid hypertrophy are found to be related to the presence of bacterial biofilms in the tonsils. This finding was largely implicated by the study of Diaz *et al.* (2011) where they found that tonsillar tissue sections obtained from patients with chronic and recurrent tonsillitis with evidence of bacterial biofilms showed increased number of lymphatic follicles in comparison to sections from patients with OSA. Therefore, prevention of a biofilm infection should be focused on in the early stages, having in mind the connection with clinical symptoms demonstrated here.

5.6 Biofilm Formation Ability of Clinical Isolates

The association between biofilm formation and persistence was accomplished by quantitative assays like the CRA method and MTP assay. The biofilm formation ability of all of our 464 isolates was found to be significantly associated with bacterial persistence. The isolates having biofilm formation ability were more likely to persist in the nasopharynx than non-biofilm formers showing a clonal distribution of strains

within the population. These findings provide evidence indicating the relationship between biofilm formation and the persistence of bacteria. Our results emphasize the need to evaluate the potential for biofilm formation before designing preventive and therapeutic strategies.

Among all our tonsillar bacterial isolates obtained from both tonsillar swabs and biopsies, 232 (50%) isolates were showed to be strong to moderate biofilm formers via MTP assay. This was further confirmed by the biofilm formation ability via CRA method where 207 (44.6%) isolates where biofilms formers as assessed by the CRA method indicating a correlation between the two methods. This was similar to results by Drago *et al.* (2012) were they evaluated the ability of bacterial isolates recovered from tonsillar biopsies and swabs to form biofilms *in vitro*. Their results showed that 44.7% of the intraoperative collected samples were either moderate or strong biofilm producers compared with 27% of isolates at 6 months after surgery concluding that the ability to form biofilm decreased in bacteria isolated after tonsillectomy which suggests a role for biofilm in pathogenesis of recurrent and chronic pharyngeal diseases such as tonsillitis (Drago *et al.*, 2012). However, our findings were in contrast to the study by Lizcano *et al.* were they tested the ability of 30 invasive and 22 non-invasive clinical isolates of *S. pneumoniae* serotype 6A and 6B to form early biofilms in polystyrene microtiter plates (Lizcano *et al.*, 2010). They suggested that the ability to form early biofilms *in vitro* does not reflect virulence potential.

The recovery of important pathogens like *Staphylococcus aureus, Haemophilus influenzae* and GABHS and their strong ability to form biofilms in vitro does suggest the existence of polymicrobial infections in tonsillar diseases and emphasize the role of bacterial load in the core of tonsils as important new concepts in understanding the development of chronic infections in otolaryngology.

The failure of conventional culture techniques to detect the antimicrobial susceptibility of bacteria in biofilms part of the failure to eradicate biofilm-associated infections. Many investigators have used the MTP assay to examine the mechanisms that contribute to biofilm formation and bacterial attachment to abiotic surfaces among pneumococcal isolates (Allegrucci and Sauer, 2007; Trappetti *et al.*, 2009). Advantages of this assay include simplicity, high-throughput screening and visualization of biofilm structures. Based on existing evidence supporting a role for biofilm formation during middle ear infection and nasopharyngeal colonization, we conclude that the ability to form biofilms *in vitro* might also contribute towards the ability to cause invasive biofilm-associated infections *in vivo*. Our results indicate that the MTP assay can serve as a reliable quantitative tool for comparing the adherences of different pathogenic strains which helps to confirm the pathogenic significance of adherence among clinically important isolates.

On CRA, biofilm producers or virulent strains produce black to almost black color colonies and non-producers from pink to bordeaux colored colonies on CRA. It is known that Congo red can directly interact with certain polysaccharide forming chromatic complexes. However, it appears more likely that some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies (Aricola *et al.*, 2001).

The CRA method is rather easy to perform, taking less time and it is sensitive and specific. Moreover it allows the formation of the colonies on the plate to be directly monitored for the appearance of phase variant bacteria, which can eventually be evidenced as pink spikes on the surface of dark colonies (Ziebuhar *et al.*, 1997). This is particularly significant because there is evidence suggesting that the phase variability itself should be regarded as an important virulence marker. Our results showed that CRA method was both sensitive and specific for biofilm detection in all bacterial

isolates including the most common isolate *Staphylococcus aureus*. This was similar to a study by Jain and Agarwal (2009) were the authors concluded the specificity of CRA when applied to *S. aureus* isolates. We conclude that CRA method can still be used to determine whether an isolate has the potential for biofilm production or not despite previous reports suggesting CRA as being slightly imprecise in the identification of positive isolates when compared to molecular analysis of the genes involved in biofilm production (Oliveira and Cunha Mde, 2010).

Since an early detection of biofilms from potentially pathogenic microorganisms can be essential towards prevention and management of biofilm-associated nosocomial infections (Jain and Agarwal, 2009), we believe that clinical microbiology laboratories implement CRA method and MTP assays as qualitative and quantitative screening techniques when dealing with clinical isolates suspected to be potential biofilm formers.

5.7 Discovery of Antibiofilm Activity from *Paenibacillus* Strain 139SI

A novel bacterial strain used in this study was originally discovered, isolated and characterized from agricultural soil. We proposed the name *Paenibacillus haemolyticus* based on the strong haemolytic activity and the results of 16S rRNA gene sequencing (Salmah *et al.*, 2012).

The bacterial cells were characterized as Gram-positive, facultatively anaerobic, endospore-forming bacteria with remarkable haemolytic activity when grown on Columbia agar supplemented with 5% sheep blood. Culturing the bacteria on blood agar is often considered as a screening method for the ability of those microorganisms to produce biosurfactants on hydrophilic media (Plaza *et al.*, 2006; Schulz *et al.*, 1991; Youssef *et al.*, 2004). Although the method has some limitations, i.e. poor specificity and diffusion restriction of the surfactant compound that may inhibit formation of

clearing zones (Jain *et al.,* 1991; Schulz *et al.,* 1991), it is still regarded as a preliminary screening method that should be supported by other identification techniques (Mulligan *et al.,* 1984). The use of enrichment media is important in providing favourable growth conditions for the organisms of interest and unfavourable for the competing organisms (Bento *et al.,* 2005; Sen, 2010; Willumsen and Karlson, 1997). In addition, sequencing of 16S rRNA gene is considered the method of choice for the accurate identification of microorganisms (Sintchenko, 2010) because it allows the study of slow-growing and uncultivable bacteria which in turn paves the way for the discovery of new bacterial species. We are in the process of obtaining the bacteriological code validation for our *Paenibacillus haemolyticus*.

In recent years, many researchers have focused on acute toxicity study on antimicrobial metabolites isolated from different soil microorganisms such as *Streptomyces* species (Bari *et al.,* 2006; Khondkar *et al.,* 1997) for the purpose of identifying new sources of bioactive natural products. Our results of acute toxicity showed that the metabolite compound isolated from novel species of *Paenibacillus haemolyticus* strain 139SI is not a toxic compound with no substantial effect on the biochemical parameters and haematological profiles of SD rats in addition to not showing any effects on cellular structures which makes us conclude that the compound can be considered potential candidate to establish new therapeutics. These findings were similar to another study made by Akhand *et al.* (2010) where they assessed the effect of a new compound from a new soil *Streptomyces* species. Their finding demonstrated that their compound did not show any effect on Long Evan's rats at a dose of 300 µg/rat/day. Thus the findings of this investigation would give valuable support to further studies and clinical trials of isolated compounds and thus new compounds with biological activity (Clarridge, 2004; Hall *et al.,* 2003; Woo *et al.,* 2008).

In our study, *Paenibacillus* possessed antibiofilm activity against clinical bacterial isolates. However, this novel species was not virulence in mice (Mahsa, 2013). Whether this bacterium can also be found in animal gut or whether there is symbiosis with its host remains to be seen. Our findings suggest that extracts from 139SI bacterial strain exhibit potent antibiofilm activity against clinically important pathogens and that treatment with this extract significantly prolonged the survival time of the experimental animal groups with no detected toxicity.

The great potential of soil microbial resource can only be truly utilize through the combination of the innovative techniques of both biology and chemistry. Therefore, we have applied HPLC profiling couples with LC-MS to identify to purified bioactive compounds from a microbial origin. This was similar to the approach applied by Mitova *et al.*, where they used HPLC bioactivity profiling/microtiter technique in conjunction with capillary NMR instrumentation in addition to AntiMarin database for the isolation and characterization of new bioactive metabolites from New Zealand fungi particularly the strain *Sepedonium chrysospermum* (Mitova *et al.*, 2008).

5.8 Therapeutic Effects of *Paenibacillus* Strain 139SI against Biofilms

In our animal model, treatment with the crude culture filtrate of *Paenibacillus haemolyticus* strain 139SI along with the synthetic furanones as a control drug significantly increased the survival time of infected rats, assisted bacterial clearance by the host and reduced the lung pathology. The rats were infected with *Pseudomonas aeruginosa* containing a large amount of an endotoxin which has been shown previously to enhanced oxidative burst response (Kharazmi *et al.*, 1991). It is likely that the metabolites produced by novel *Paenibacillus haemolyticus* strain 139SI may activate the endotoxin-primed neutrophils.

This was similar to the study made by Wu *et al.*, where they tested synthetic furanones for their ability to attenuate bacterial virulence in the mouse models of chronic lung infection by targeting bacterial quorum-sensing without directly killing bacteria or inhibiting their growth (Wu *et al.*, 2004). They reported that furanones successfully interfered with N-acyl homoserine lactone and suppressed bacterial quorum-sensing in lungs, which resulted in an accelerated lung bacterial clearance, and a reduced severity of lung pathology.

Inhibition of the alginate barrier produced by *P. aeruginosa* using nontoxic compounds targeted against alginate biosynthetic may be useful in eliminating *P. aeruginosa* biofilms from chronically infected tissues such as the lungs of CF patients (May *et al.*, 1991). It was noticed that the areas with pathologic changes in treatment group was smaller than that in the positive control group. The incidence of chronic inflammation in treatment group including 6 (12.5%) rats was lower than that in control group 12 (25%) (Table 4.7). This was similar to the findings made by Song *et al.*, when they evaluated the effect of ginseng treatment on the oxidative burst response of peripheral blood neutrophils and alveolar macrophages in a rat model of chronic mucoid *P. aeruginosa* lung infection (Song *et al.*, 1998).

The fact that majority of rats classified under Score II, i.e. mild focal inflammation of the lung, were belonging to the comparative control group 5 (10.4%) and the treatment group 6 (12.5%) whereas majority of rats under Score IV, i.e. severe inflammation to necrosis of the lung, belong to the positive control group 10 (20.83%) indicates the therapeutic effect of 139SI filtrate and 2(5H)-Furanone in attenuating the biofilm infection of *Pseudomonas aeruginosa* in the lungs of SD male and female rats.

PMNs are among the blood cells that are important for the host defense against bacterial infections. However, in CF patients the infiltration of numerous PMNs in the lung tissues affected with chronic *P. aeruginosa* infection does not clear the bacteria

effectively. Instead, the PMNs become an important cause of tissue damage of the lung parenchyma because of the release of lysosomal enzymes, particularly leukocyte elastase (Høiby, 1995). It has been shown that the extracellular products of *P. aeruginosa*, alkaline protease and elastase, can inhibit the myeloperoxidase- mediated chemiluminescence which is one of the major antimicrobial systems manifested by PMNs (Kharazmi *et al.*, 1984) This might be among the reasons that PMN infiltration into lungs affected by CF does not eliminate the *P. aeruginosa* infection effectively. Another reason is the weak response of PMNs to the biofilm mode of growth of *P. aeruginosa* in the lungs of CF patients (Høiby *et al.*, 1995).

Our finding suggests that culture filtrate from novel bacterial species of *Paenibacillus haemolyticus* strain 139SI bacterial strain exhibited antibiofilm activity against clinically important pathogens and that treatment with this extract significantly prolonged the survival time of the experimental animal and was able to clear the artificial alginate that mimics the biofilm environment. However, the identified compounds were not further evaluated to identify their chemical structure and antibiofilm activity. The mechanism of antibiofilm activity for *Paenibacillus haemolyticus* strain 193SI are yet to be explored.

5.9 Conclusions

This study contributes important findings that have not been reported by others where compounds derived from the novel bacterial species of *Paenibacillus haemolyticus* strain 193SI showed remarkable antibiofilm activity against clinically important pathogens and was capable of inhibiting the biofilm formation of a broad range of bacterial isolates recovered from chronically infected tonsils which adds an important new dimension to the search for potent drugs against bacterial biofilm

infections. Other findings conclude that ENT infections such as chronic and recurrent tonsillitis are biofilm-related and the strong ability of clinical bacterial isolates that are susceptible to antibiotics to being strongly associated with biofilm formation. In addition to the ability in establishing an animal model of chronic lung infection that is treated with crude filtrate of metabolites produced by the novel *Paenibacillus haemolyticus* strain 139SI.

More studies are needed to identify the mechanism(s) of action of these antibiofilm agents and characterize the diversity of the genus *Paenibacillus* and their adaptation to the soil environment secondary metabolite production will create a better understanding of the potential utility of these bacteria as a source of useful products for medicine particularly against biofilm infections.

5.10 Limitations of the Study

1. No control group due to difficulty in obtaining tonsils from age-matched individuals who never had infection or obstruction in the upper airways (Kania *et al.*, 2007).
2. No assessment on the role of anaerobic bacteria, viruses and fungi in tonsillar diseases due to the difficulties in collecting, transporting and culturing them.
3. No pre and post tonsillectomy assessment of infant's development and body weight (Greenfeld *et al.*, 2003).
4. No post-tonsillectomy evaluation of patients' quality of life (QOL) (Witsell *et al.*, 2008).
5. No evaluation between biofilm in tonsillar diseases among ethnic groups (no racial comparison study).
6. It was not possible to establish a rat model for tonsillitis because rodents have a different pharyngeal anatomy than humans and they lack both the palatine and nasopharyngeal tonsils (Perry and Whyte, 1998).

5.11 Strengths of the Study

1. The discovery of a secondary metabolite compound from a taxonomically novel species of *Paenibacillus* that exhibits antibiofilm activity against a wide range of clinically important pathogens.

2. The use of Confocal Laser Scanning Microscopy as a standard tool to detect the presence of bacterial biofilm in the palatine tonsils.

3. To avoid selection (sample) bias, patients were clinically assessed by the ENT specialists and medical officers at UMMC prior to inclusion to ensure adequate representation of the specimens.

4. To avoid measurement bias, tonsillar specimens (i.e. swabs and biopsies) were referred to the Clinical Diagnostic Laboratories (CDL) at University Malaya Medical Centre (UMMC) for proper isolation and identification of bacterial isolates.

5. To avoid information bias, characterization and identification of the antibiofilm compounds from 139SI filtrate were blindly assessed by a certified consultancy group using skilled technicians equipped with highly calibrated equipment.

5.12 Future Studies

1. Evaluation of the immunopathology of tonsils with and without biofilms.
2. A racial comparison study for the presence of biofilms among paediatric patients with OSA.
3. Post-tonsillectomy quantification of the oropharyngeal microbial flora and their biofilm formation among larger sample size.
4. Post-operative assessment of the effectiveness of tonsillectomy in eradicating biofilm infections.
5. Establishment of a rational basis in the treatment of tonsillar diseases by comparing the pre-operative symptoms with the respective changes that took place post-operatively.
6. Administration of 139SI compound in rats as a goggle or spray rather than orally.
7. Evaluation of the *in vivo* antibiofilm activity of the FR5 potential compound.
8. Identify the mechanisms of action of all the potential 139SI antibiofilm compounds.

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APPENDIXES

Appendix 1 Awards Obtained with this Study

1A Gold Medal (Innovation & Creativity Expo 2010)

UNIVERSITY OF MALAYA
The Leader in Research & Innovation

INNOVATION & CREATIVITY '10 EXPO
UNIVERSITY OF MALAYA
1 - 3 APRIL 2010

INNOVATION AND CREATIVITY EXPO 2010
UNIVERSITY OF MALAYA
1-3 APRIL 2010

CERTIFICATE OF AWARD

Gold Medal

for outstanding achievement in
Student Gallery
(Sciences)

presented to
Saad Musbah Naji Alasil
Salmah Ismail
Mahmood Ameen

in recognition of research excellence titled
Discovery of Novel Properties from Bacterial Culture Supernatant via a Rapid and Simple Method towards Developing Anti-Quorum Sensing and Anti-Biofilm Drugs

Pacific Ocean
Hong Kong
Manila

GHAUTH JASMON
Vice-Chancellor
University of Malaya

1B Best of Category Award (Innovation & Creativity Expo 2010)

UNIVERSITY OF MALAYA
The Leader in Research & Innovation

INNOVATION & CREATIVITY '10 EXPO
UNIVERSITY OF MALAYA 1 - 3 APRIL 2010

INNOVATION AND CREATIVITY EXPO 2010
UNIVERSITY OF MALAYA
1-3 APRIL 2010

CERTIFICATE OF AWARD

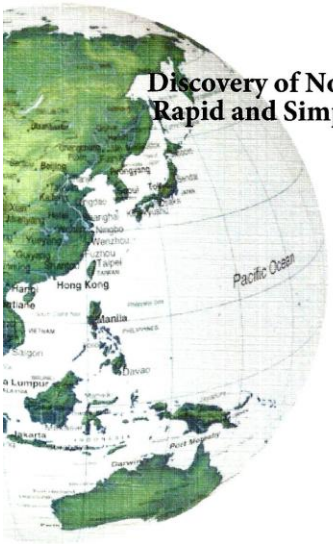
Best of Category

for outstanding achievement in

Student Gallery
(Science)

presented to

Rahmat bin Omar
Saad Musbah Naji Alasil
Salmah binti Ismail
Mahmood Ameen Abdulla Hassan



in recognition of research excellence titled

Discovery of Novel Properties from Bacterial Culture Supernatant via a Rapid and Simple Method Towards Developing Anti-Quorum Sensing and Anti-Biofilm Drugs

GHAUTH JASMON
Vice-Chancellor
University of Malaya

1C Silver Medal (International Invention, Innovation & Tech Exhibition 2010)

MINISTRY OF SCIENCE, TECHNOLOGY & INNOVATION

ITEX MALAYSIA — INTERNATIONAL INVENTION, INNOVATION AND TECHNOLOGY EXHIBITION

MINDS

Certificate of Award

This is to certify that

ASSOC. PROF. DR. RAHMAT OMAR, DR. SALMAH ISMAIL,
SAAD MUSBAH NAJI ALASIL,
ASSOC. PROF. DR. MAHMOOD AMEEN ABDULLA HASSAN

has been awarded the

ITEX SILVER MEDAL

for the invention

BIOFILM PROFILE OF CHRONIC AND RECURRENT TONSILLITIS USING VARIOUS MICROSCOPIC TECHNIQUES AND THEIR OUTCOME ON THE DIAGNOSIS AND TREATMENT OF BIOFILM-RELATED INFECTIONS

at the

21st International Invention, Innovation & Technology Exhibition
ITEX 2010
Kuala Lumpur, Malaysia

held from

14th – 16th May 2010

Academician Emeritus Professor Tan Sri Datuk
Dr Augustine Ong Soon Hock
President

Appendix 2 Participations Associated with this Study

2A Innovation & Creativity Expo 2010, University Malaya

UNIVERSITY OF MALAYA
The Leader in Research & Innovation

INNOVATION & CREATIVITY '10 EXPO
UNIVERSITY OF MALAYA
1 - 3 APRIL 2010

UNIVERSITY OF MALAYA

Certificate of Participation

This certificate is awarded to

Saad Musbah Naji Alasil
Salmah Ismail
Mahmood Ameen
Rahmat Omar

for your participation in the

INNOVATION AND CREATIVITY EXPO 2010
UNIVERSITY OF MALAYA

on

1-3 APRIL 2010

at

DEWAN TUNKU CANSELOR,
UNIVERSITY OF MALAYA

GHAUTH JASMON
Vice-Chansellor
University of Malaya

2B International Invention, Innovation & Technology Exhibition, 2010

MINISTRY OF SCIENCE, TECHNOLOGY & INNOVATION

ITEX MALAYSIA — INTERNATIONAL INVENTION, INNOVATION AND TECHNOLOGY EXHIBITION

MINDS

Certificate of Participation

This is to certify that

ASSOC. PROF. DR. RAHMAT OMAR,
DR. SALMAH ISMAIL, SAAD MUSBAH NAJI ALASIL,
ASSOC. PROF. DR. MAHMOOD AMEEN ABDULLA HASSAN

showcased the invention

BIOFILM PROFILE OF CHRONIC AND RECURRENT TONSILLITIS USING VARIOUS MICROSCOPIC TECHNIQUES AND THEIR OUTCOME ON THE DIAGNOSIS AND TREATMENT OF BIOFILM-RELATED INFECTIONS

at the

21st International Invention, Innovation & Technology Exhibition
ITEX 2010
Kuala Lumpur, Malaysia

held from

14th - 16th May 2010

Academician Emeritus Professor Tan Sri Datuk
Dr. Augustine Ong Soon Hock
President
Malaysian Invention and Design Society

2C Biological Sciences Graduate Congress, 2010

UNIVERSITY OF MALAYA
KUALA LUMPUR

Bridging Ideas, Building Talents

Certificate of Participation

This is to certify that

Saad Musbah Naji Alasil
(Poster Presenter)

Has participated in

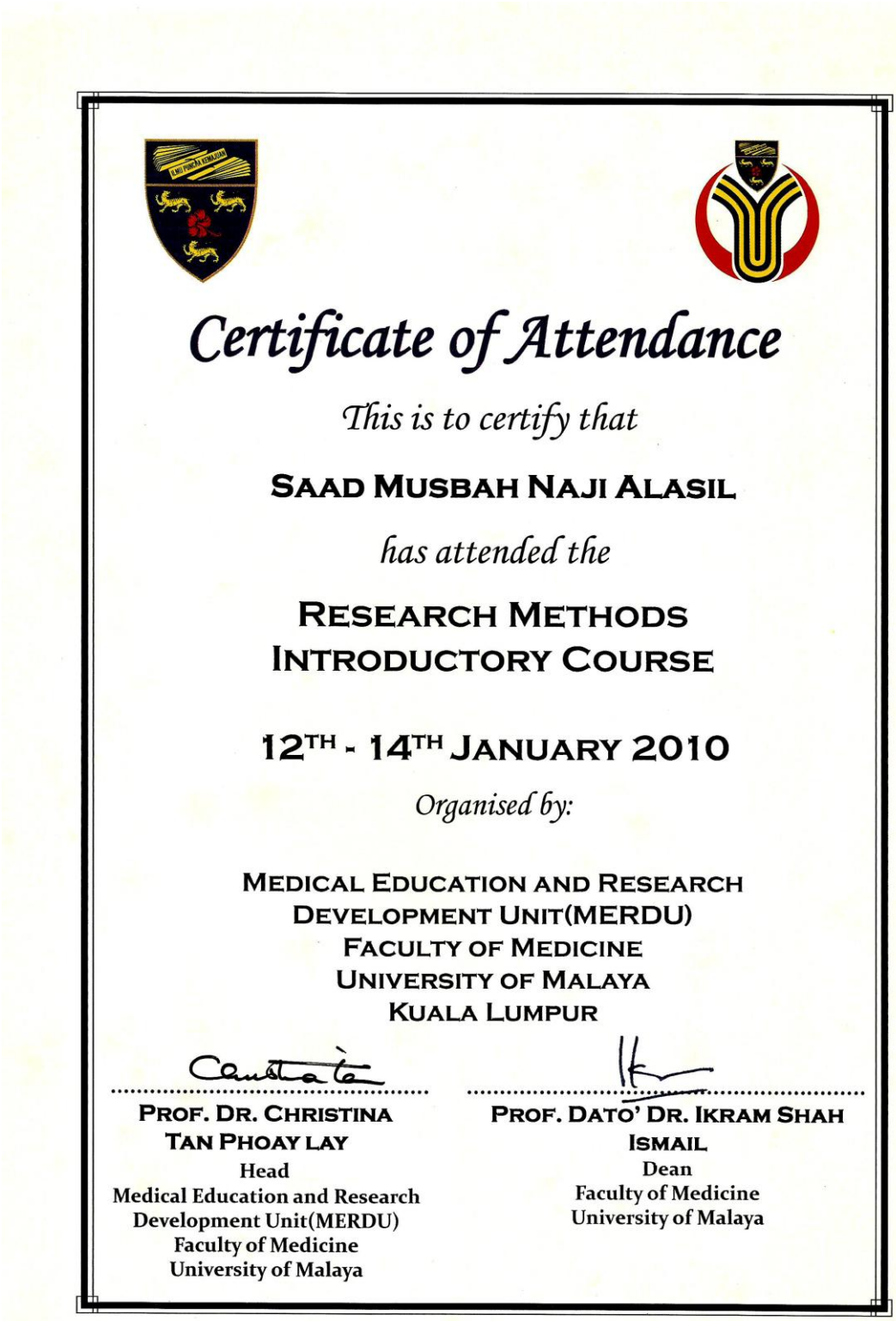
The 15th Biological Sciences Graduate Congress 2010

15th -17th December 2010

Institute of Biological Sciences, Faculty of Science
University of Malaya,
Kuala Lumpur, MALAYSIA

Prof. Dato' Dr. Mohd Sofian Azirun
Dean
Faculty of Science
University of Malaya
Kuala Lumpur, MALAYSIA

2D Research Methods Introductory Course, University of Malaya



Certificate of Attendance

This is to certify that

SAAD MUSBAH NAJI ALASIL

has attended the

RESEARCH METHODS INTRODUCTORY COURSE

12TH - 14TH JANUARY 2010

Organised by:

MEDICAL EDUCATION AND RESEARCH DEVELOPMENT UNIT(MERDU)
FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

PROF. DR. CHRISTINA TAN PHOAY LAY
Head
Medical Education and Research Development Unit(MERDU)
Faculty of Medicine
University of Malaya

PROF. DATO' DR. IKRAM SHAH ISMAIL
Dean
Faculty of Medicine
University of Malaya

2E Confocal LSM 700 Workshop, University of Malaya

UNIVERSITI MALAYA
KUALA LUMPUR

ZEISS

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA

Certificate Of Attendance

SAAD MUSBAH NAJI

Confocal LSM 700 Workshop In UM

11 FEBRUARY 2011

Jointly organized by

Electron Microscope Unit
&
Carl Zeiss Sdn.Bhd.

PROF MADYA NAZARINA ABDUL RAHMAN
HEAD OF ELECTRON MICROSCOPE UNIT,
DEPARTMENT OF MEDICINE,
UNIVERSITY OF MALAYA

DR SAMUEL KO,
DEMONSTATOR,
CARL ZEISS SDN.BHD.

2F Course on Basics and Principles of HPLC, University of Malaya

SUCXeS

(SHIMADZU-UMMC CENTRE FOR XeNOBIOTICS STUDIES)
University Malaya Medical Centre

Certificate of Attendance

This is to Certify that

SAAD MUSBAH NAJI ALASIL

has attended the course

Basics and Principles of High Performance Liquid Chromatography (HPLC) in Biomedical Analysis

conducted by SUCXeS on 13th to 14th December 2010
at the Faculty of Medicine, University of Malaya, Kuala Lumpur.

Professor Dr. Mustafa Ali Mohd
Head and Senior Consultant,
Shimadzu-UMMC Centre for Xenobiotics Studies,
Faculty of Medicine,
University of Malaya, Kuala Lumpur.

UNIVERSITY OF MALAYA
KUALA LUMPUR
FACULTY OF MEDICINE

2G Course on Bacterial Genomics, Malaysia Genomics Recourse Centre

MALAYSIAN GENOMICS RESOURCE CENTRE BERHAD

is proud to present

Saad Musbah Naji Alasil

with this

Certificate of Attendance

for participating in MGRC's Premium Workshop Series titled

Bacterial Genomics

From Biology to Publication

held on

10-11 April 2013

at

MGRC Training Centre
Mid Valley City
Kuala Lumpur

Robert George Hercus @ Abdul Karim Hercus
Founder and Managing Director

Appendix 3 Publications Arising from this Study

3A African Journal of Microbiology Research, 2011

African Journal of Microbiology Research Vol. 5(27), pp. 4748-4752, 23 November, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.024

Full Length Research Paper

Bacterial identification and antibiotic susceptibility patterns of *Staphyloccocus aureus* isolates from patients undergoing tonsillectomy in Malaysian University Hospital

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Diagnosis and treatment of chronic tonsillitis (CT) and recurrent tonsillitis (RT) are common problems seen in any otolaryngology clinic. Antibiotic therapy usually fails to prevent the recurrences of these infections leaving the patient with no choice but surgery (tonsillectomy). This study aims to identify the bacteriology of both tonsillar swabs and biopsies specimens among children and adult patients diagnosed with CT and RT (infected tonsils). In addition to obstructive sleep apnea (OSA) and tonsillar hypertrophy (TH) (non-infected tonsils) all undergoing elective tonsillectomy and to characterize the antibiotic susceptibility patterns of the most common isolate. A total number of 138 surgically removed palatine tonsils were collected from 72 patients. Six types of antibiotics were selected to test the susceptibility of *Staphylococcus aureus* isolates which are methicillin, gentamicin, erythromycin, co-trimoxazole, clindamycin and fusidic acid. The most common isolate was *S. aureus* (190 isolatre) followed by *Haemophilus influenza* (83), *Streptococcus* Group B (61), *Haemophilus parainfluenza* (33), *Klebsiella pneumoniae* (32), *Streptococcus* Group G (29), *Streptococcus* Group F (14), *Streptococcus* Group C (12), *Pseudomonas aeruginosae* (10) and Streptococcus Group A (9) and *Streptococcus* Group A (9). The number of *S. aurues* isolates in infected tonsils (RT and CT) was 145 (76.3%) while in non-infected tonsils (OSA and TH) was 45 (23.6%). Three susceptibility patterns were found among *S. aureus* isolates in all cases, (89.4%) susceptible to all the selected antibiotics, (10.6%) resistant to fusidic acid only, whereas 0.5% resistant to both methicillin and fusidic acid. There was a 30.5% difference in the type of bacterial isolates between tonsillar swab and biopsies. The high prevalence of *S. aureus* in patients with both infected and non-infected tonsils suggests that this bacterium might not be the aetiological agent of chronic and recurrent tonsillitis. In addition, the high susceptibility rate of methicilin among *S. aurues* isolates could be due to the fact that *S. aurues* represents part of the oropharynx normal flora. The increased rate of resistance 10.6% to fusidic acid among methicillin-susceptible *S. aureus* (MSSA) isolates makes this antibiotic a less potential drug of choice for patients with chronic and recurrent tonsillitis.

Key words: Antibiotic susceptibility, *Staphylococcus aureus*, tonsillectomy, swab and biopsy.

INTRODUCTION

The diagnosis and treatment of chronic and recurrent tonsillitis has been a common problem to otolaryngologist (Logantham et al., 2006). In the past, chronic tonsillitis was largely a clinical concept but today bacteriological and patho-anatomical considerations are getting more attention (Upal and Bais, 1989). Despite the fact that tonsillitis is frequent, a general agreement to identify the main causative microorganisms and their differences

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between children and adults is still lacking (Logantham et al., 2006). It has been reported that bacteria causing tonsillitis are present in both tonsillar surface and deep tissue.

However, the isolates taken from surface may not always reflect the real pathogens (Inci et al., 2002). *Staphylococcus aureus* is among the leading Gram positive bacteria that can be found in the normal flora of oropharynx and nose (Shanmugam et al., 2008). In recurrent tonsillitis (RT) the tonsillar core contains a large number of bacteria including *S. aureus, Haemophilus influenza* and *Streptococcus pyogenes* in which there is a strong correlation between the type of isolate and the tonsillar infection (Lindroos, 2000).

Antibiotic therapy usually fails to prevent the recurrence of chronic tonsillitis (CT) and recurrent tonsillitis (RT) because inappropriate usage against the pathogen in deep tonsillar tissue leads to the continuation of infection and re-inoculation causing recurrence leaving the patient with no choice but surgery (Mostafa et al., 2009).

Even though clinical cases with infected tonsils like RT and CT are different with non-infected tonsils like obstructive sleep apnea (OSA) and tonsillar hypertrophy (TH) in terms of the course of disease and symptoms, they share a common aspect of surgery (tonsillectomy) as a treatment of choice.

The bacteriology of tonsils among RT, CT and TH patients has been previously reported by Andreas et al. (2010); Kurien et al. (2000) and Lindroos (2000), respectively. In our study we added the clinical case of OSA in order to identify the bacteriology of tonsillar swabs and biopsies and to characterize the antibiotic susceptibility patterns of the most common isolate among patients undergoing elective tonsillectomy at University of Malaya Medical Center (UMMC). Determining the bacteriology of tonsillar biopsy (core) is important for several reasons because failure to identify and eradicate pathogens in the core will allow persistence of core infection or re-inoculation of initially sterilized surface.

MATERIALS AND METHODS

Patient selection

This is a cross sectional study performed on 72 patients diagnosed with four types of clinical cases undergoing elective tonsillectomy. Infected tonsils were collected from RT and CT cases while non-infected tonsils from OSA and TH for a period of 9 months (October 2009 to June 2010) at the University of Malaya Medical Center (UMMC) situated in Kuala Lumpur, Malaysia. Upon approval from UMMC Medical Ethics Committee (PPUM/UPP/300/02/02Ref. No. 744.11), a written consent was taken from all the selected patients. The age of patients to be considered as adults was more than 12 years. Inclusion criteria included three or more severe recurrent attacks of tonsillitis in two consecutive years (Loganathan et al., 2006). Patients are considered to have severe illness if at least three associated symptoms, that is high fever, snoring during acute attacks, unable to take normal diet, absence from school and work, and admission to hospital, are present. However, unilateral enlargement, pregnancy and immune compromised patients were considered as exclusion criteria (Loganathan et al., 2006).

Specimen collection

The selected patients undergoing general anesthesia were followed by retraction of the uvula and soft palate (Figure 1). Swabs were taken from the surface of palatine tonsils with a sterile cotton-tipped applicator then placed in a transport medium. Tonsillectomy was performed and biopsies were collected and rinsed with phosphate buffered saline. Biopsy specimens were cut into appropriate size using surgical scalpel before being kept in a sterile labeled container in order to be processed. All specimens were sent immediately to the microbiology unit of Clinical Diagnostic Laboratory (CDL) at UMMC.

Bacterial identification

Biopsies were placed in a sterile Petri dish and minced until complete homogenization with nutrient broth before being cultured onto blood agar (BA), chocolate agar (CA), MacConkey's agar (Mac), fastidious anaerobic agar (FAA) and thioglycollate broth. All the plates were incubated for 24 to 48 h according to the type of media used. For *S. aureus*, isolates were Gram stained and further characterized using coagulase and DNAse tests. Non *S. aureus* isolates including *Streptococcus* species were identified via Optochin test, bile solubility test and bacitracin tests; Gram negative bacilli via kliger's iron agar, indole, citrate, malonate utilization, urease, oxidase, methyl red (MR) and voges-proskauer (VP) tests; and *Haemophilus* species via XV factor test.

Disk diffusion test

Susceptibility of *S. aureus* isolates was determined by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar. Bacterial colonies from each isolate were transferred into a suspension medium adjusted to 0.5 McFarland turbidity standards (1.5 × 108 CFU/ml). Inoculums were swabbed on the entire surface of agar plates followed by the application of six selected commercially available antibiotic disks of methicillin (30 µm), gentamicin (10 µm), erythromycin (15 µm), co-trimoxazole (1.25 + 23.75 µm), clindamycin (30 µm) and fusidic acid (10 µm) using sterile forceps (5 disks per plate). Plates were inverted and incubated for 18 to 24 h at 37°C. The antibiotic vancomycin was not included in the susceptibility testing of methicillin-resistant *S. aureus* (MRSA) isolates. Zones of inhibition were determined according to the standards outlined by the Clinical and Laboratory Standards Institute (CLSI) (Cockerill et al., 2010).

Statistical analysis

Statistical significance differences of *S. aureus* susceptibility between age and gender groups were assessed using one-way ANOVA for which all values were reported as standard error mean (S.E.M) ±. A probability value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Based on our results, 10 types of bacterial isolates were identified from 138 surgically removed tonsils of 72 patients. The age of patients ranged from 4 to 38 years with a mean of 14.8 years. Adult female patients

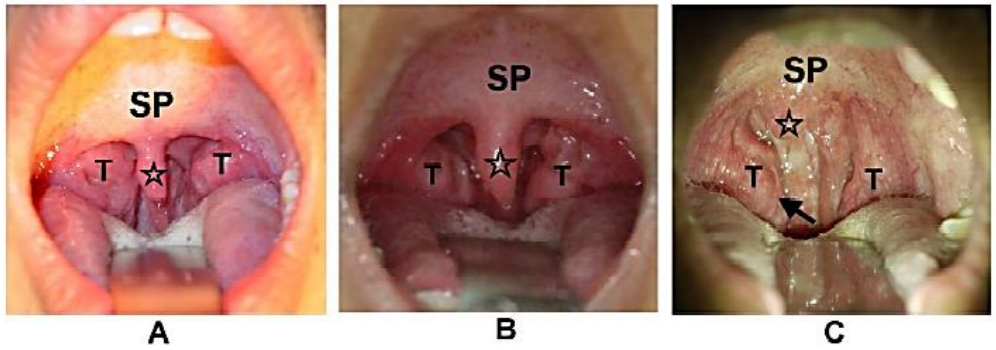

Figure 1. (A) Tonsillar enlargement due to recurrent tonsillitis. (B) Tonsillar enlargement in Obstructive Sleep Apnoea. (C) Unilateral/asymmetric tonsillar hypertrophy. (T= tonsil, SP- soft palate, star- uvula).

Table 1. Prevalence of clinical cases among patients.

Clinical diagnosis	Children		Adult		Total		
	Male count (%)	Female count (%)	Male count (%)	Female count (%)	Male count (%)	Female count (%)	All count (%)
Recurrent Tonsillitis (RT)	9 (12.5)	12 (16.6)	8 (11.1)	20 (27.7)	17 (23.6)	32 (44.3)	49 (68)
Chronic Tonsillitis (CT)	3 (5.5)	0 (0.0)	1 (1.3)	3 (4.05)	4 (6.8)	3 (4.05)	7 (9.8)
Obstructive Sleep Apnea (OSA)	4 (5.5)	2 (2.7)	0 (0.0)	4 (5.5)	4 (5.5)	6 (8.3)	10 (13.8)
Tonsillar Hypertrophy (TH)	2 (2.7)	2 (2.7)	1 (1.3)	1 (1.3)	3 (4.05)	3 (4.05)	6 (8.4)

were the most dominant in all clinical cases 28 (38.8%) followed by 19 children male patients (26.3%), 16 children female (22.2%) and 10 adult male (13.8%). Prevalence of clinical cases according to gender and age groups is shown in Table 1. *S. aureus* was the most common bacteria (190 isolates) in both infected and non-infected tonsils followed *H. influenza* (83), *Streptococcus* Group B (61), *H. parainfluenza* (33), *Klebsiella pneumoniae* (32), *Streptococcus* Group G (29), *Streptococcus* Group F (14), *Streptococcus* Group C (12), *Pseudomonas aeruginosae* (10) and *Streptococcus* Group A (9). Among all 72 patients, only 14 did not show *S. aureus* isolates from their tonsillar specimens. The number of *S. aurues* isolates in infected tonsils (RT and CT) was 145 (76.3%) while in non-infected tonsils (OSA and TH) was 45 (23.6%).

Three susceptibility patterns were found among *S. aureus* isolates in all cases, (89.4%) susceptible to all the selected antibiotics, (10.6%) resistant to fusidic acid only whereas 0.5% resistant to both methicillin and fusidic acid. Susceptibility of *S. aureus* isolates and their distribution are shown in Table 2. There was no significance in the difference of susceptibility between swab and biopsy specimens among adults 2.00 ± 0.23, children 2.18 ± 0.98, male 1.92 ± 0.916 and female 2.37 ± 0.74 in all the four clinical cases.

Table 2. Susceptibility patterns of *S. aureus* isolates and their distribution among age groups.

Susceptibility pattern	Children		Adult		Total		
	Swab count (%)	Biopsy count (%)	Swab count (%)	Biopsy count (%)	Swab count (%)	Biopsy count (%)	All count (%)
Susceptible	37 (19.4)	39 (20.5)	45 (23.6)	48 (25.2)	82 (43.1)	87 (45.7)	169 (89)
Resistant	4 (2.06)	8 (4.1)	5 (2.6)	4 (2.06)	9 (4.7)	12 (6.18)	21 (11)

There was a 30.5% difference in the type of bacterial isolates between tonsillar swab and biopsies.

DISCUSSION

The reason we have selected UMMC as a site for our study is because it represent a tertiary care hospital in addition to the fact that antibiotic resistance among bacteria varies between different geographic areas even within the same country (Lim, 2003). Donnelly et al. (1994) found that recurrent tonsillitis was the commonest indication for tonsillectomy. Same observation was found with our study. In a previous study, the most frequent isolate from tonsillar surface was found to be *Streptococcus pneumoniae* (66.6%) followed by group A β-hemolytic streptococci (62.5%), *S. aureus* (38.4%) and *H. influenzae* (27.2%) (Mustafa et al., 2007). In contrast, Gaffney et al. (1991) reported that *H. influenza* was the most common isolate from tonsillar core followed by *S. aureus*. Kumar et al. (2005), reported *S. aureus* as the common isolate from both tonsillar surface and core followed by β-haemolytic streptococci. However, in our study we have showed that *S. aureus* was the most frequent isolate in both tonsillar swabs and biopsies among all the four clinical cases followed by *H. influenza* then *Streptococcus* Group B with no significance between age and gender which gives an indication that the type of tonsillar microorganisms may vary due to the antibiotic regiments that are being prescribed in that clinical setting. This highlights the importance of monitoring tonsillar bacteriology using reliable diagnostic techniques. Determining the bacteriology of tonsillar biopsy (core) is important because failure to identify and eradicate pathogens in the core, whether it is from inappropriate antibiotic choice or from insufficient penetration into the core, will allow persistence of core infection or re-inoculation of initially sterilized surface (Kurein et al., 2000).

A study by Brook and Foote in "Microbiology of normal tonsils" concluded that there is polymicrobial flora in normal and infected tonsils but their number and encapsulation is increased in inflammatory process (Brook and Foote 1990). Our study showed that *S. aureus* was found in 33.2% of children with CT and RT (infected tonsils) compared to 13.6% children with OSA and TH (non-infected tonsils). In contrast, *H. influenzae* was found in 32% of children with recurrent tonsillitis compared to 48% of children without tonsillar disease (Bista et al., 2006).

The correlation between chronic and recurrent tonsillitis and the presence of *S. aurues* in both children and adults was very strong yet the high prevalence of *S. aureus* in patients with both infected and non-infected tonsils suggests that this bacterium might not be the aetiological agent of chronic and recurrent tonsillitis.

The high susceptibility rate of methicilin among *S. aureus* isolates could be due to that fact that *S. aureus* is part of the oropharynx normal flora. On the other hand it is emphasized that among any large population of *S. aureus* isolates, resistance to fusidic acid will occur (Mette et al., 1990) and development of fusidic acid resistance during therapy has been described especially when it was used systemically as a single agent (Pattison et al., 1973). Fusidic acid is recommended to be used as combination therapy with clindamycin, rifampicin or trimethoprim (Rohani et al., 2000). Its usage as combination therapy did not lead to the development of resistance (Rosdahl, 1988).

In Malaysian hospitals, the resistance rates for fusidic acid and rifampicin individually were reported to be between 3 to 5% in the years 1992 to 1996 as these antibiotics represent an alternative therapy to vancomycin in Malaysia (Norazah et al., 2002). Similarly in our study, an increased rate of resistance to fusidic acid was found to be 10.6% among methicillin-susceptible *S. aureus* (MSSA) isolates which suggests making it a less potential drug of choice for patients with chronic and recurrent tonsillitis. A similar finding was reported among MSSA isolates in Bristol with a resistance to fusidic acid increasing from 6% in 1998 to 11.5% in 2001 due to the frequent use of this antibiotic in topical applications (Brown and Thomas 2002). Educating the medical staff on the appropriate prescription of fusidic acid and rifampicin represents an important step to prevent further emergence of these resistant strains.

The reason why patients with chronic and recurrent tonsillitis do not respond to antibiotic therapy remains unclear. Few explanations could be due to the low concentrations of antibiotics in the tonsillar core due to the presence of scar tissue after each infection causing less diffusion of the antibiotic into the core (Usamah et al., 2005). In addition to the presence of normal flora that produces protective enzymes and the formation of biofilms (Andreas et al., 2010). Further studies are needed to assess the etiological agent of the infected and non-infected tonsils among larger sample size to eliminate the discrepancies between surface and biopsy (core) isolates.

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Paenibacillus hemolyticus, the first hemolytic *Paenibacillus* with growth-promoting activities discovered

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Abstract: *Paenibacillus spp.* are Gram-positive, facultatively aerobic, bacilli-shaped endospore-forming bacteria. They have been detected in a variety of environments, such as soil, water, forage, insect larvae, and even clinical samples. The strain 139SI (GenBank accession No.: JF825470.1) from three strains of *Paenibacillus* isolates investigated here was chosen as the type strain of the proposed novel species. The other two similar strain isolates investigated were 140SI (JF825471.1) and 141SI (JQ734548.1). These strains were identified as members of the genus *Paenibacillus* on the basis of phenotypic characteristics, phylogenetic analysis and 16S rRNA G+C content. Surprisingly, these strains exhibited a strong hemolytic activity on 5% sheep blood agar. Their crude extracts also showed positive growth-promoting activities in colon cancer and Vero cell lines. To our knowledge, this is the first *Paenibacillus* with hemolytic and growth-promoting activities reported, and the name *Paenibacillus hemolyticus* for this novel species is proposed. The capability of this novel species in hemolytic and cell growth activities suggests its potential in both clinical and pharmacological implications.

Key words: *Paenibacillus hemolyticus*; soil bacteria; hemolytic; anticancer and cytotoxic activities; 16S rRNA G+C content.

Abbreviations: A_t, absorbance of test sample; BHI, brain-heart-infusion; CRA, Congo red agar; dNTPs, deoxynucleotide triphosphates; DSMZ, German Collection of Microorganisms and Cell Cultures; %G+C, guanine plus cytosine content in percentage; HCT, hematocrit; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCBI, National Center for Biotechnology Information; SI, Salmah Ismail.

Introduction

A group of anaerobic, Gram-positive and endospore-forming bacteria were initially classified under the genus *Bacillus*, based on their phenotypic characteristics. Later, phylogenetic analyses using 16S rRNA with 11 identified species isolated from various habitats re-classified them as a separate genus, *Paenibacillus* (Ash et al. 1993). To date, there are 137 species of *Paenibacillus* approved and validated according to the bacterial nomenclature list by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Braunschweig (DSMZ), Germany (URL: http://www.dsmz.de/download/bactnom/bactname.pdf; March 2012). The genus *Paenibacillus* has been isolated from various ecological habitats, including warm springs (Saha et al. 2005; Chou et al. 2007; Akel et al. 2008), alkaline soils (Yoon et al. 2005), and agricultural soil (Ma & Chen 2008). Various properties were reported among *Paenibacillus* species, including xylanolytic (polysaccharide-degradation), nitrogen fixation and proteases (Berge et al. 2002; Hrabák & Martínek 2007). Actual March 2012, no hemolytic activity had been reported for *Paenibacillus spp.* According to DSMZ.

Here, we report on the identification and characterization of a novel *Paenibacillus* species that is not only capable to show remarkable hemolytic activity, but it also exhibits growth promotion in colon cancer and Vero cell lines. For the first time, strains of *Paenibacillus* possessing both hemolytic and growth-enhancing activities with potential clinical implications are reported.

Material and methods

Colony isolation

Strains Salmah Ismail (SI), 139SI (GenBank accession No.: JF825470.1; ATCC BAA-2268); 140SI (JF825471.1) and 141SI (JQ734548.1) were originally isolated from an agricultural soil obtained from Serdang Agricultural Center,

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Selangor, Malaysia. This soil sample was thoroughly suspended in 3 mL of sterile distilled water, the suspension was streaked on brain-heart infusion (BHI) agar plates supplemented with 5% sheep blood, and plates were incubated for 16–24 h at 37 °C. Hemolytic colonies obtained from the plates were sub-cultured again on BHI blood agar and incubated under aerobic conditions. Strains 139SI, 140SI and 141SI were among the isolates that appeared to exhibit a strong hemolytic activity (β-hemolysis) and were routinely cultured on BHI blood agar and maintained as a glycerol suspension (25%, w/v) at −80 °C and BHI-slant agar at room temperature. Of 141 isolates of *Paenibacilli* and *Bacilli* from the soil extract 52% showed negative hemolytic activities on BHI blood agar and hence were used as negative controls against SI strains. To supplement the hemolytic property, the biosurfactant activity assay for the crude protein filtrates from strains 139SI, 140SI and 141SI was also conducted using drop collapsing test (Youssef et al. 2004) and the positive emulsification activity was observed as milky-colored smear indicating that the oil and filtrate were miscible.

Bacterial Gram identification

A test for bacterial Gram differentiation was performed using Gram staining and non-staining methods of an in-house alkaline lysis and aminopeptidase strip test (Merck KGaA, Darmstadt, Germany). For the alkaline lysis method, a solution of 1% sodium dodecyl sulfate and 0.2 N NaOH was introduced to the overnight bacterial culture of strains SI prior to two min incubation at 4 °C. Aminopeptidase strip test was carried out as described previously (Farmer 2005). The Gram-negative and Gram-positive control isolates used were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, respectively.

Biochemical tests and phenotypic characterizations

Biochemical identification tests including catalase, oxidase, urease, glucose, sucrose, indole, mannitol and lactose were performed as described by Cappuccino & Sherman (2011). Growth at three temperatures (25 °C, 37 °C and 50 °C) was assessed after 2–3 days incubation. Salt tolerance was tested on BHI agar medium supplemented with 1–10% (w/v) NaCl after 2–3 days incubation. Caseinase test was performed by inoculating one streak of inoculum on BHI agar supplemented with skimmed milk. The plates were inverted and incubated at 37 °C for 24–48 h for zone inhibition observation around and below the colonies. Additionally, plates were inoculated on nutrient agar, trypticase soy agar and MacConkey agar and incubated aerobically for 24–48 h at 37 °C to evaluate their growth properties.

Congo red agar (CRA) method

To detect the virulence (slime production) of the cultured strains, CRA method was carried out according to the protocol originally described by Freeman et al. (1989). The tested strains were cultured on CRA plates prepared by adding 0.8 g of Congo red (Sigma) and 36 g of saccharose (Sigma) to 1 L of BHI agar (Bio-Rad). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121 °C for 15 min and was added when the agar had cooled to 55 °C. Plates were incubated at room temperature (37 °C) for 24 h under aerobic conditions and followed overnight at room temperature (Mathur et al. 2006; Snoussi et al. 2008). The Congo red dye directly interacts with certain polysaccharides, forming colored complexes (Jain & Agarwal 2009). A five-color reference scale was used to accurately determine all color variations shown by the colonies. Virulent strains will form black colonies on CRA, whereas a non-virulent strain will form red colonies. Colonies exhibiting a bright black and dry opaque black morphology were classified as virulent strains (biofilm producers), whereas red, pink and bordeaux colonies were classified as non-virulent strains (non-biofilm producers) (Oliveira & Cunha 2010).

Microscopic observation of cellular morphology

Cellular morphology was observed under scanning electron microscopy using an overnight culture of strains SI grown in BHI broth. To prepare the sample for scanning electron microscope, 1 mL of pure bacterial culture was transferred into a 1 mm × 1 mm rounded filter paper and air dried for 10 min. The sample was rinsed in 0.1 M phosphate buffer, followed by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature for 4 h. The cells were dehydrated by using series of ethanol concentrations: 30%, 50%, 70%, 85%, 95% and 100%, and then two times with 100% acetone. Critical point drying was done by Hitachi HCP-2 critical point dryer. The samples were then mounted on specimen stubs and sputter-coated with gold. The samples were observed by Zeiss DSM 950 scanning electron microscope at 15 kV (Ko et al. 2007).

Investigating the cytological effects of the novel Paenibacillus on cell cultures

The colon cancer HCT 116 cell line for anticancer experiments and Vero cell line for cytotoxic experiments were prepared as described previously (Wilson 2000). Cell suspension of 5,000 cells/well was adjusted to the 96 well microtitre plate. The filtrate (crude extract) of soil bacterial 139SI and 140SI strains grown overnight in BHI agars was obtained by centrifugation at 10,000 rpm for 10 min at 4 °C. Filtrate was passed through a 0.22 µm filter (Millipore, USA) to remove any existing bacterial pellets or particles. Ten µL of 139SI and 140SI crude extracts at 56, 112, 224, 448, 896 µg of protein contents, and distilled water as a control, were added to the wells separately followed by 48 h incubation. The anticancer (HCT 116 cell line) and cytotoxic (Vero cell line) activities were then determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Mosmann 1983; Wilson 2000). The MTT colored formazan product absorbance was measured using a microplate reader at 490 nm. The percentage of cell viability was calculated using the following formula:

$$\% \text{ cell viability} = (A_t - A_b)/(A_c - A_b) \times 100$$

where A_t – absorbance value of test sample, A_b – absorbance value of blank, and A_c – absorbance value of control. The data were expressed as the mean ± standard error (S.E.) using Microsoft Excel 2007 software.

16S rRNA sequencing identification

The strain 139SI (JF825470.1) from three strains of *Paenibacillus* isolates was chosen as the type strain of the proposed novel species. Thus, for further confirmation of the 139SI isolate, molecular identification using sequencing of 16S rRNA was performed as described previously (Aye et al. 2011). Strain 139SI was cultured on nutrient agar plates (Oxoid Ltd., Hampshire, UK) at 37 °C overnight. Following the incubation, bacterial colonies were picked with sterile wooden tooth picks suspended in sterile milli-Q water (Millipore, Molsheim, France) and boiled for 5 min. The suspension was then centrifuged at 12,000×*g* for 10 min and the resulting supernatant containing the bacterial DNA (50–100

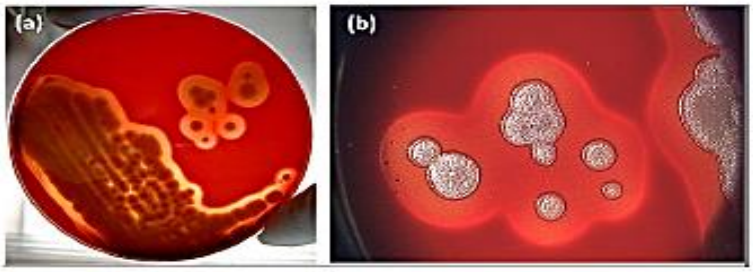

Fig. 1. A representative picture of strong hemolysis by strain 139SI on 5% sheep blood agar after 16 h incubation at 37°C. (a) Underneath view, (b) on top view.

ng) was used as template for the PCR amplification (Misbah et al. 2005).

Amplification of the 16S rDNA was performed in a DNA thermal cycler, PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) with the following cycling program: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The bacterial DNA was amplified using three sets of primers, synthesized by Research Biolabs Pte Ltd. (Singapore). All reactions were performed in 25 µL volumes, containing 15 pmol of each primer, PCR nucleotide mix (dNTPs), Mg-free buffer, $MgCl_2$ and Taq DNA polymerase (Promega, Madison, USA). The amplified DNA fragments were gel-purified using QIAquick™ GelExtraction Kit (250) (Qiagen, Hilden, Germany) and sequenced by Macrogen Inc. (Seoul, Korea) using an ABI3730 XL DNA Analyser (Applied Biosystems, Renton, USA). Sequencing results from each amplified fragment were aligned and assembled into contigs to obtain a complete 16S rDNA consensus sequence using Sequencher™ ver. 4.0.5 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequencing of the amplified 16S rRNA gene was carried out prior to phylogenetic analysis of SI strains (Yoon et al. 1998).

Phylogenetic analysis of SI strains

The data from 16S rRNA gene sequence of the strains 139SI (JF825470.1; ATCC BAA-2268), 140SI (JF825471.1) and 141SI (JQ734548.1) were compared with available 16S rRNA gene sequences from GenBank database (Benson et al. 2010) using the nucleotide BLAST (Altschul et al. 1990) program (http://www.ncbi.nlm.nih.gov/BLAST/). Determination of phylogenetic positions of strains SI among the neighboring species of the genus *Paenibacillus* based on 16S rRNA gene sequences (Baek et al. 2010) was carried out according to the neighbor-joining method (Saitou & Nei 1987) followed by 1,000 replications of bootstraping using the software MEGA 4 (Tamura et al. 2007). In addition, the G+C content in percentage (%G+C) of all the *Paenibacillus* records from National Center for Biotechnology Information (NCBI) was calculated using the Mobyle@Pasteur server (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::geecee) and the frequency of species against the %G+C distribution was computed using an in-house R-program (R Development Core Team 2008). The distribution pattern was identified and used to validate the *Paenibacillus* genus for strains SI using the G+C content.

Results and discussion

Biochemical and morphological identification

The isolated colonies were grey, large, rough and irregular edged with a size of 2–3 mm in diameter exhibiting a strong hemolytic activity after 16 h incubation at 37°C on 5% sheep blood agar (Fig. 1). In addition, milky smears were observed for the drop collapsing test for biosurfactant production by the crude filtrate from strains 139SI, 140SI and 141SI (strains SI) confirming the co-existence of hemolytic activity and biosurfactant production of these bacteria (Dehghan-Noudeh et al. 2005). Cells of strains SI were confirmed as Gram-positive rod-shaped with bipolar end terminal spores. No lysis of bacterial cell wall was observed in alkaline lysis solution, while no change in the color of bacterial suspension was detected by the strip of aminopeptidase test indicating a Gram-positive reaction. Strains SI exhibited a positive reaction to glucose, sucrose and oxidase tests, whereas a negative reaction to urease, indole, lactose and mannitol. Optimal growth temperature was found to be 37°C and no growth was observed at 50°C. Growth was observed in the presence of 0–6% (w/v) NaCl on BHI agar and 0–4% (w/v) NaCl on nutrient agar at 37°C. Clear zones were detected around and below the colonies grown on BHI skimmed milk agar plate indicating a caseinase-positive activity. Growth was observed on nutrient agar, trypticase soy agar, but not MacConkey agar. The colonies of strains SI were pink and bordeaux on CRA, which was similar to the result of *E. coli* ATCC 25922 indicating a non-virulent strain in comparison to methicillin resistant *S. aureus*, which exhibited pigmented black colonies after 24 h incubation. All cells were observed as regular rods with a size of 0.4–0.5 µm × 2.0–2.5 µm (Fig. 2).

Growth-promoting activities of strains 139SI and 140SI crude extracts

Experiments on cell cultures using strains 139SI and 140SI show that increased concentration of crude extracts is accompanied with the enhancement of cell viability (Table 1). In anticancer experiments, initial crude extract of 139SI shows lower percentage viability com-

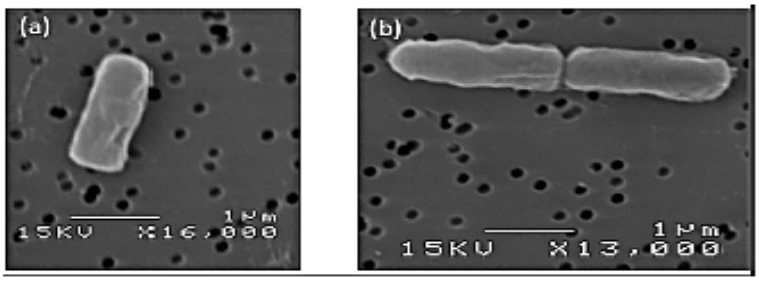

Fig. 2. A representative of scanning electron micrograph of a cell of strain 139SI after growth for 48 h in Brain Heart Infusion broth. (a) A single regular *Bacillus*, (b) two cells of strains 139SI; bar 1 μm.

Table 1. Anticancer and cytotoxic activities of strains 139SI and 140SI against the colon cancer HCT 116 cell line and Vero cell line, respectively.[a]

Crude proteins [μg]	Anticancer activity [% viability][b]	Cytotoxic activity [% viability][b]
139SI		
56	37.2 ± 4.0	122.5 ± 8.7
112	57.5 ± 8.0	126.9 ± 13.0
224	67.0 ± 6.2	130.0 ± 10.5
448	72.1 ± 8.9	133.6 ± 7.9
896	86.5 ± 2.5	135.5 ± 7.2
140SI		
56	54.8 ± 8.7	116.5 ± 8.3
112	65.3 ± 7.2	116.7 ± 7.6
224	68.0 ± 9.9	120.1 ± 6.9
448	74.2 ± 8.3	121.1 ± 1.1
896	79.4 ± 11.3	126.7 ± 15.4

[a] The MTT coloured formazan product absorbance was measured using a microplate reader at 490 nm. All data were expressed as the mean ± standard error (S.E.) using Microsoft Excel 2007 software.
[b] The percentage of cell viability was calculated using the following formula: % cell viability = $(A_t - A_b)/(A_c - A_b) \times 100$, where A_t – absorbance value of test sample, A_b – absorbance value of blank, A_c – absorbance value of control. Distilled water was used as control with 100% cell viability.

pared to 140SI but a higher percentage viability was obtained at the highest crude extract concentration. In cytotoxic tests, crude extract from 139SI constantly shows higher percentage viability compared to 140SI. Factors that contribute to growth-promoting properties for both 139SI and 140SI extracts are still unknown. More thorough biochemical tests are required to further characterize the molecular nature of the observed "growth factors" in this novel *Paenibacillus*. Overall, our results implicate the presence of growth-transforming proteins or genetic elements in this novel *Paenibacillus* that could act as a growth-promoting agent.

Phylogenetic analyses of 139SI

Similarity calculations of the 16S rRNA gene sequence were made between the strains SI and the type strains of other members of Paenibacillaceae. The nucleotide BLAST results showed that strains SI belong to the genus *Paenibacillus* and exhibited a high similarity level of 99% with *Paenibacillus alvei* to a lower level of 95% with *Paenibacillus terrigena* in 16S rRNA gene sequence. This relationship between strains SI and other members of the genus *Paenibacillus* was also evident in the phylogenetic tree obtained with the neighbor-joining method (Fig. 3). The strains of 139SI (JF825470.1, ATCC BAA-2268) thus represents a novel species of the genus *Paenibacillus* and this suggestion is strengthened on the basis of 1–5% differences (Clarridge III 2004) with respect to recognized species in this genus. For the 16S rRNA sequences, a search at NCBI returned a total of 3,381 records of *Paenibacillus* species. The %G+C against the frequency of species gave an almost normal distribution curve with a mean of 55.0 ± 1.3 %G+C. Our result of %G+C for strains SI was 54.1 ± 0.3. From the distribution curve shown in Figure 4, strains SI were certain to be *Paenibacillus* at a confidence level of 97.7% at 2σ based on the 16S rRNA %G+C. Thus, the 16S rRNA G+C content proved to be useful to identify a *Paenibacillus* genus with a characteristic G+C content of 55.0 ± 1.3% by computing all the 3,381 *Paenibacillus* 16S rRNA records as a mini 16S rRNA library from the NCBI.

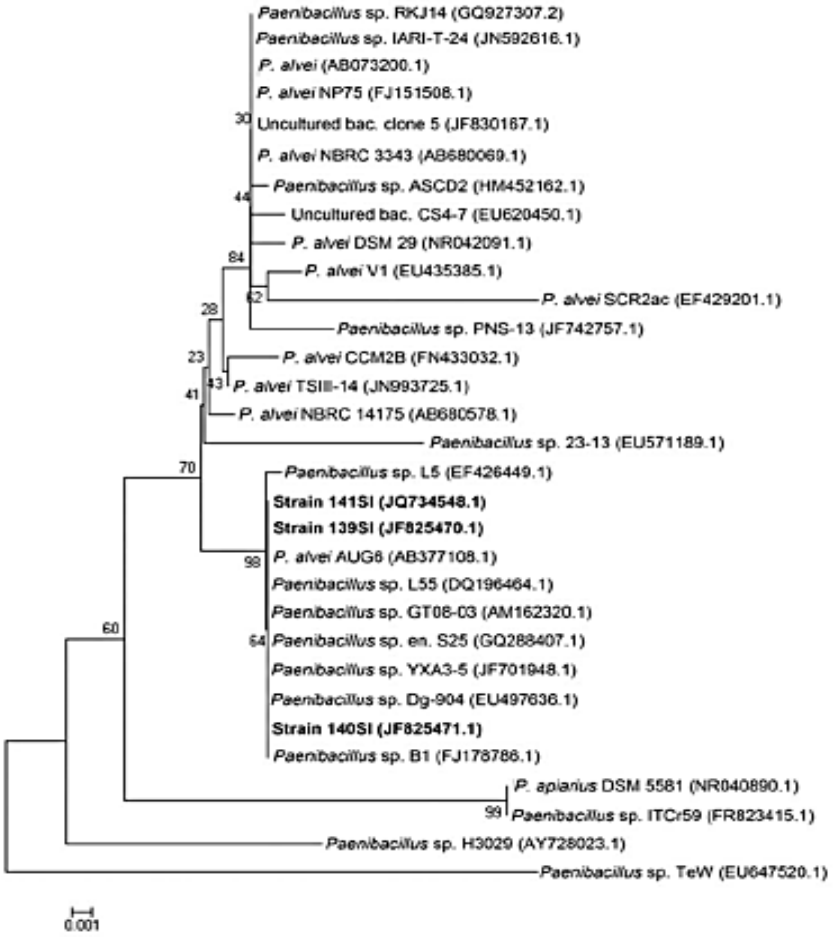

Fig. 3. Phylogenetic positions of strains Salmah Ismail (SI): comparative analysis of the 16S rRNA gene sequences of strains SI and the type strains of phylogenetically related representatives of the genus *Paenibacillus* based on the neighbour-joining method followed by 1,000 replications of bootstraping. Strains SI are in bold.

Paenibacillus hemolyticus sp. nov. as the proposed name for the novel species identified

The bacterial cells are Gram-positive, facultatively anaerobic, endospore-forming bacteria and straight rods of 0.4–0.5 μm × 2.0–2.5 μm in size. Colonies grown on BHI-blood agar are grey, large, rough, and irregular edged with a size of 2–3 mm in diameter exhibiting a strong hemolytic activity. Growth occurs on BHI agar, nutrient agar, but not on MacConkey agar and the optimum growth occurs at 37°C. It grows in the presence of 0–6% (w/v) NaCl on BHI agar and 0–4% (w/v) NaCl on nutrient agar. The isolates were identified as members of a novel species of the genus *Paenibacillus* based on their hemolytic activities and 16S rRNA gene sequences. According to the phenotypic characteristics, combination of strong hemolytic activity, biochemical tests and negative growth on MacConkey agar, it is apparent that strains SI were not affiliated to any recognized species of the genus *Paenibacillus*. Since these strains were clearly distinguished from all other *Paenibacillus* species on the basis of the data presented, it is considered that strains SI represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus hemolyticus* sp. nov. is proposed.

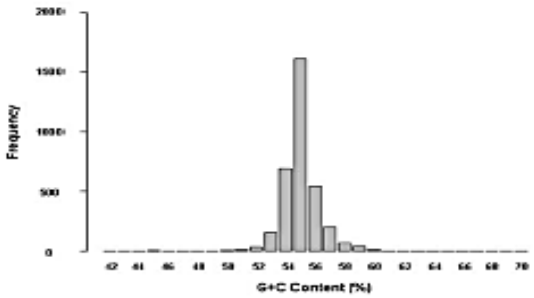

Fig. 4. The distribution of the frequency of *Paenibacillus* sp. against the G+C percentage plotted by an in-house R-program from a total of 3,381 NCBI records of *Paenibacillus* species. The plotted values show an almost normal distribution curve.

Potential clinical importance of Paenibacillus hemolyticus

A few members of *Paenibacillus*, in particular *P. alvei*, had been reported to cause human disease (Ash et al. 1993). A more recent case study also revealed that another member of this genus, *Paenibacillus thiaminolyticus*, an environmental bacterium which is initially thought to play no role in human disease, had been reported to cause infection in human (Ouyang et al. 2008). These studies implicate potential clinical cases for other members of *Paenibacillus*. In this work, we identified the novel species *Paenibacillus hemolyticus* showing strong activity in hemolysis and growth-promoting activities on cell cultures, suggesting its potential role in tumorigenesis. However, this novel species did not show virulent activity using CRA test. Furthermore, we identified this species also possesses a caseinase-positive activity. Whether this bacterium can also be found in animal gut or whether there is a symbiosis to its host remain to be seen. More future works are required to further characterize this novel bacterium, in particular its potential role in human infection and growth-transforming activities.

Acknowledgements

This study was supported by grants from the Biotechnology and Bioproduct Research Cluster (UMRG: RG026–09BIO) and Human Translational Medicine Research Cluster (RG163/09HTM) of the University of Malaya Research Grants and Research Grant Scheme (FRGS: FP026-2010B) from the Ministry of Higher Education (MOHE). The authors wish to thank Madam Mariam (retired in August, 2009) from Serdang Agricultural Center for kindly providing the soil material. We also thank the Tropical Infectious Diseases Research Center (TIDREC), Faculty of Medicine, University of Malaya for rRNA sequencing services and Clinical Diagnostic Laboratory (CDL), University Malaya Medical Center (UMMC) for biochemical tests confirmation. We are also grateful to Mr. Maysam, Mr. Pouya Hasandarwish and Ms. Siti Fatimah Zahra Mohd Anuar from Department of Molecular Medicine, Faculty of Medicine, University of Malaya for their valuable technical assistance.

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Accepted July 11, 2012

Appendix 4 Approval Letter from Medical Ethics Committee, UMMC

4A First Page of the Letter

UNIVERSITI MALAYA
KUALA LUMPUR
PUSAT PERUBATAN UM

يونيورسيتي مالايا

No. Rujukan:- PPUM/UPP/300/02/02

20 Syawal 1430H
09 Oktober 2009

Prof. Madya Rahmat Omar
Jabatan Otorinolaringologi
Pusat Perubatan Universiti Malaya

Tuan,

SURAT PEMAKLUMAN KEPUTUSAN PERMOHONAN MENJALANKAN PROJEK PENYELIDIKAN
Biofilm Study In Chronic And Recurrent Otorhinolaryngologic Infections Towards Effective Prevention And Therapeutic Management: Cloning And Expression Of Anti-Quorum Sensing Related Genes Against Its Formation
Protocol No:
MEC Ref. No: 744.11

Dengan hormatnya saya merujuk kepada perkara di atas.

Bersama-sama ini dilampirkan surat pemakluman keputusan Jawatankuasa Etika Perubatan yang bermesyuarat pada 30 September 2009 untuk makluman dan tindakan tuan selanjutnya.

2. Sila maklumkan kepada Jawatankuasa Etika Perubatan mengenai butiran kajian samada telah tamat atau diteruskan mengikut jangka masa kajian tersebut.

Sekian, terima kasih.

Yang benar,

Norashikin Mahmood
Setiausaha
Jawatankuasa Etika Perubatan
Pusat Perubatan Universiti Malaya

s.k Ketua
Jabatan Otorinolaringologi

Jawatankuasa Etika Perubatan
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izanie@ummc.edu.my

SIRIM ISO 9001:2000 · 074 REG. NO AR 2567

4B Second Page of the Letter

UNIVERSITI MALAYA
KUALA LUMPUR
PUSAT PERUBATAN UM

JAWATANKUASA ETIKA PERUBATAN
PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79494422 samb. 3209 FAKSIMILI: 03-79494636

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre ADDRESS: LEMBAH PANTAI 59100 KUALA LUMPUR	ETHICS COMMITTEE/IRB REFERENCE NUMBER: 744.11
PROTOCOL NO: TITLE: Biofilm Study In Chronic And Recurrent Otorhinolaryngologic Infections Towards Effective Prevention And Therapeutic Management: Cloning And Expression Of Anti-Quorum Sensing Related Genes Against Its Formation	
PRINCIPAL INVESTIGATOR: Prof. Madya Rahmat Omar TELEPHONE: KOMTEL:	SPONSOR: MOSTI

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[✓] Borang Permohonan Penyelidikan	Ver date: 4 Sept 09
[✓] Study Protocol	Ver date:
[ ] Investigator's Brochure	Ver date:
[✓] Patient Information Sheet	Ver date:
[✓] Consent form	Ver date:
[ ] Questionnaire	
[✓] Investigator(s) CV's (Prof. Madya Rahmat Omar)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

i. *Investigator is required to follow instructions, guidelines and requirements of the Medical Ethics Committee.*
ii. *Investigator is required to report any protocol deviations violations through the Clinical Investigation Centre and provide annual/closure reports to the Medical Ethics Committee.*

Date of approval: 30th SEPTEMBER 2009

s.k Ketua
Jabatan Otorinolaringologi

Timbalan Dekan (Penyelidikan)
Fakulti Perubatan, Universiti Malaya

Setiausaha
Jawatankuasa Penyelidikan Pusat Perubatan
Fakulti Perubatan, Universiti Malaya

PROF. LOOI LAI MENG
Chairman
Medical Ethics Committee

4C Third Page of the Letter

UNIVERSITI MALAYA
KUALA LUMPUR
PUSAT PERUBATAN UM

JAWATANKUASA ETIKA PERUBATAN
PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79494422 samb. 3209 FAKSIMILI: 03-79494638

MEDICAL ETHICS COMMITTEE COMPOSITION, UNIVERSITY MALAYA MEDICAL CENTRE
Date: 30th September 2009

Member (Title and Name)	Occupation (Designation)	Male/Female (M/F)	Tick (✓) if present when above items were reviewed
Chairperson: Prof. Looi Lai Meng	Representative Dean/Director	Female	✓
Deputy Chairperson: Prof. Kulenthran Arumugam	Consultant Medical Education Research and Development Unit (MeRDU)	Male	✓
Secretary (non- voting): Cik Norashikin Mahmood	Science Officer, Medical Development Unit	Female	✓
Members: 1. Prof. Jamiyah Hassan	Deputy Chairman (Professional)	Female	
2. Prof. Nor Zuraida Zainal	Head of Department of Psychological Medicine	Female	✓
3. Assoc. Prof. Mohamed Ibrahim Noordin	Head of Department of Pharmacy, FOM	Male	
4. Prof. Tan Chong Tin	Representative Head Of Department Of Medicine	Male	
5. Assoc. Prof. George Lee Eng Geap	Representative Head of Department of Surgery	Male	✓
6. Assoc. Prof. Grace Xavier	Lecturer, Faculty of Law	Female	✓
7. Tuan Haji Amrahi b. Buang	Senior Manager, PTj Farmasi UMMC	Male	✓
8. YBhg. Datin Aminah Pit Abdul Rahman	Public Representative	Female	✓
9. Madam Ong Eng Lee	Public Representative	Female	

Comments: The MEC of University Malaya Medical Centre is operating according to ICH GCP guideline and the Declaration of Helsinki. Members no. 6, 8 & 9 are representatives from Faculty of Law in the University of Malaya and the public, respectively. They are independent of the hospital or trial site.

PROF. LOOI LAI MENG
Chairman
Medical Ethics Committee

Appendix 5 Consent Form by Responsible Relative

5A English Version of the Form

UNIVERSITY MALAYA MEDICAL CENTRE

CONSENT BY RESPONSIBLE RELATIVE FOR CLINICAL RESEARCH

I, ...Identity Card No.....
(Name)

of.....
(Address)

hereby agree that my relative..... I.C. No.....
(Name)
participate in the clinical research (clinical study/questionnaire study/drug trial) specified below:-

Title of Study: **CLONING AND EXPRESSION OF ANTI-QUORUM SENSING RELATED GENES FOR EFFECTIVE PREVENTION AND THERAPEUTIC MANAGEMENT AGAINST BIOFILMS FORMATION IN OTORHINOLARYNGOLOGIC CHRONIC AND RECURRENT INFECTIONS.**

the nature and purpose of which has been explained to me by **A/P DR. RAHMAT OMAR** and interpreted
(Name & Designation of Doctor)

by **SAAD MUSBAH NAJI ALASIL** to the best of his ability in.....language/dialect.
(Name & Designation of Interpreter)

I have been informed of the nature of this clinical research in terms of procedure, possible adverse effects and complications (as per patient information sheet). I understand the possible advantages and disadvantages of participating in this research. I voluntarily give my consent for my relative to participate in this research specified above.

I understand that I can withdraw my relative from this clinical research at any time without assigning any reason whatsoever and in such situation; my relative shall not be denied the benefits of usual treatment by the attending doctors. Should my relative regains his/her ability to consent, he/she will have the right to remain in this research or may choose to withdraw.

Date: ..... Relationship to Patient ..... Signature or Thumbprint .....

IN THE PRESENCE OF

Name .....)

Identity Card No. .....) Signature .....
(Witness)

Designation .....)

I confirm that I have explained to the patient's relative the nature and purpose of the above-mentioned clinical research.

Date ..... Signature .....
(Attending Doctor)

CONSENT BY RESPONSIBLE RELATIVE FOR CLINICAL RESEARCH

R.N.
Name
Sex
Age
Unit

5B Malay Version of the Form

UNIVERSITY MALAYA MEDICAL CENTRE

KEIZINAN OLEH WARIS YANG BERTANGGUNGJAWAB UNTUK PENYELIDIKAN KLINIKAL

Saya,..... Kad Pengenalan .....
(Nama Waris yang bertanggungjawab)

beralamat.....
(Alamat)

dengan ini bersetuju supaya saudara saya..... menyertai
(Nama Pesakit)

dalam penyelidikan klinikal (pengajian klinikal/pengajian soal-selidik/percubaan ubat-ubatan) disebut berikut:

Tajuk Penyelidikan: **CLONING AND EXPRESSION OF ANTI-QUORUM SENSING RELATED GENES FOR EFFECTIVE PREVENTION AND THERAPEUTIC MANAGEMENT AGAINST BIOFILMS FORMATION IN OTORHINOLARYNGOLOGIC CHRONIC AND RECURRENT INFECTIONS.**

yang mana sifat dan tujuannya telah diterangkan kepada saya oleh **A/P DR. RAHMAT OMAR**
(Nama & Jawatan Doktor)

mengikut terjemahan **SAAD MUSBAH NAJI ALASIL** yang telah menterjemahkan kepada saya dengan
(Nama & Jawatan Penterjemah)

sepenuh kemampuan dan kebolehannya di dalam.....Bahasa / loghat

Saya telah diberitahu bahawa dasar penyelidikan klinikal dalam keadaan metodologi, risiko dan komplikasi (mengikut kertas maklumat pesakit). Saya mengetahui dan memahami semua kemungkinan kebaikan dan keburukan penyelidikan klinikal ini. Saya merelakan/mengizinkan saudara saya menyertai penyelidikan klinikal tersebut di atas.

Saya faham bahawa saya boleh menarik balik penyertaan saudara saya dalam penyelidikan klinikal ini pada bila-bila masa tanpa memberi sebarang alasan dalam situasi ini dan tidak akan dikecualikan dari kemudahan rawatan dari doktor yang merawat. Sekiranya saudara saya kembali berupaya untuk memberi keizinan, beliau mempunyai hak untuk terus menyertai kajian ini atau memilih untuk menarik diri.

Tarikh: ..... Pertalian dengan Pesakit ..... Tandatangan/Cap Jari Waris yang bertanggungjawab .....

DI HADAPAN

Nama .....

No. K/P.....) Tandatangan .....
(Saksi untuk Tandatangan Waris yang Bertanggungjawab)

Jawatan.....)

Saya sahkan bahawa saya telah menerangkan kepada waris yang bertanggungjawab sifat dan tujuan penyelidikan klinikal tersebut di atas.

Tarikh: ..... Tandatangan .....
(Doktor yang merawat)

KEIZINAN OLEH PESAKIT
UNTUK
PENYELIDIKAN KLINIKAL

No. Pend.
Nama
Jantina
Umur
Unit

Appendix 6 Certificate of Deposit *Paenibacillus haemolyticus* strain 139SI at ATCC

BACTERIOLOGY PROGRAM

A global bioscience nonprofit
Organization dedicated to biological
Standards and biodiversity

American Type Culture Collection
10801 University Boulevard
Manassas, Virginia 20110-2209 USA
Internet http://www.atcc.org

CERTIFICATE OF DEPOSIT

June 15, 2011

Paenibacillus haemolyticus Strain 139SI (ATCC BAA-2268) was deposited in the ATCC general collection of bacteria by Salmah Ismail, University of Malaya, Kuala Lumpur, Malaysia, on May 11th, 2011;

The strain was confirmed to be viable and pure. The genotypic/phenotypic characteristics of this strain were determined to be consistent with those provided by the depositor.

This culture is available for immediate distribution. Researchers may request the organism using the ATCC catalog number. Information on ordering the strain can be accessed through our website at www.atcc.org.

Brian J. Beck

Brian J. Beck, Ph.D.
Bacteriology Program Manager
American Type Culture Collection (ATCC)
10801 University Boulevard
Manassas, VA 20110-2209

Phone: (703) 365-2700 ext. 2676
Fax: (703) 365-2730
Internet: http://www.atcc.org

Appendix 7 Accession Number of *Paenibacillus* sp. 16S rRNA gene Sequence at GenBank

Paenibacillus sp. 139SI 16S ribosomal RNA gene, partial sequence

GenBank: JF825470.1

FASTA Graphics PopSet

Go to:

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DEFINITION  Paenibacillus sp. 139SI 16S ribosomal RNA gene, partial sequence.
ACCESSION   JF825470
VERSION     JF825470.1  GI:350285761
KEYWORDS    .
SOURCE      Paenibacillus sp. 139SI
  ORGANISM  Paenibacillus sp. 139SI
            Bacteria; Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus.
REFERENCE   1  (bases 1 to 1496)
  AUTHORS   Salmah,I., Saad,A.M., Siti-Fatimah-Zahra and Rahmat,O.
  TITLE     DNA Sequence of 16S rRNA from a novel 139SI strain of Paenibacillus
            sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1496)
  AUTHORS   Salmah,I., Saad,A.M., Siti-Fatimah-Zahra and Rahmat,O.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-APR-2011) Molecular Medicine, University of Malaya,
            Jalan Pantai, Kuala Lumpur, WP 50603, Malaysia
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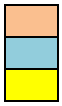
Appendix 8 List of Patients with their Clinical Cases, Type and Date of Surgery

Patient No.	Sex	Age	Clinical Case	Type of Operation	Date of Surgery
1	F	7	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	26/10/2009
2	M	11	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	26/10/2009
3	M	23	Recurrent Tonsillitis	Tonsillectomy	26/10/2009
4	F	9	Recurrent Adenotonsillitis	Tonsillectomy & Adenoidectomy	02/11/2009
5	M	16	Recurrent Tonsillitis	Tonsillectomy	04/11/2009
6	M	31	Recurrent Tonsillitis	Tonsillectomy	09/11/2009
7	M	16	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	09/11/2009
8	M	16	Chronic Tonsillitis	Tonsillectomy	18/11/2009
9	M	11	OSA Secondary to Recurrent Acute Tonsillitis	Tonsillectomy & Adenoidectomy	30/11/2009
10	F	11	Recurrent Tonsillitis	Tonsillectomy	02/12/2009
11	M	5	Recurrent Tonsillitis	Tonsillectomy	07/12/2009
12	F	9	Recurrent Tonsillitis	Tonsillectomy	07/12/2009
13	F	13	Recurrent Tonsillitis with Bilateral MEE	Tonsillectomy & Adenoidectomy	09/12/2009
14	M	9	Recurrent Tonsillitis with Bilateral MEE	Tonsillectomy & Adenoidectomy	09/12/2009
15	F	26	Recurrent Tonsillitis	Tonsillectomy	14/12/2009
16	F	24	Recurrent Tonsillitis	Tonsillectomy	28/12/2009
17	F	26	Recurrent Tonsillitis	Tonsillectomy	13/01/2010
18	F	17	Recurrent Tonsillitis with Bilateral MEE	Tonsillectomy & Adenoidectomy	18/01/2010
19	F	15	Recurrent Adenotonsillitis	Tonsillectomy & Adenoidectomy	27/01/2010
20	F	10	Recurrent Tonsillitis	Tonsillectomy	03/02/2010
21	M	8	Recurrent Tonsillitis	Tonsillectomy	03/02/2010
22	F	5	Recurrent Tonsillitis with Snoring	Tonsillectomy & Adenoidectomy	10/02/2010
23	F	5	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	10/02/2010
24	M	6	Recurrent Tonsillitis	Tonsillectomy	10/02/2010
25	F	17	Chronic Tonsillitis	Tonsillectomy	22/02/2010
26	M	7	Obstructive Sleep Apnea	Tonsillectomy & Adenoidectomy	01/03/2010
27	F	28	Recurrent Tonsillitis	Tonsillectomy	03/03/2010
28	M	6	OSA Secondary to Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	08/03/2010
29	F	18	Chronic Tonsillitis with Snoring	Tonsillectomy	08/03/2010
30	F	24	Chronic Tonsillitis	Tonsillectomy	10/03/2010
31	F	13	Recurrent Tonsillitis	Tonsillectomy	15/03/2010

32	F	12	Recurrent Tonsillitis with Bilateral MEE	Tonsillectomy	15/03/2010
33	F	9	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	22/03/2010
34	M	8	OSA Secondary to Tonsillar Hypertrophy	Tonsillectomy	22/03/2010
35	F	23	Recurrent Tonsillitis	Tonsillectomy	22/03/2010
36	F	24	Recurrent Tonsillitis	Tonsillectomy	29/03/2010
37	M	11	Chronic Tonsillitis	Tonsillectomy	31/03/2010
38	M	9	Recurrent Tonsillitis	Tonsillectomy	31/03/2010
39	F	10	Recurrent Tonsillitis	Tonsillectomy	31/03/2010
40	F	27	Recurrent Tonsillitis	Tonsillectomy	12/04/2010
41	F	16	Obstructive Sleep Apnea	Tonsillectomy & Adenoidectomy	12/04/2010
42	F	8	Obstructive Sleep Apnea	Tonsillectomy & Adenoidectomy	14/04/2010
43	M	10	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	14/04/2010
44	M	8	Chronic Tonsillitis	Tonsillectomy	19/04/2010
45	M	4	OSA Secondary to Tonsillar Hypertrophy	Tonsillectomy & Adenoidectomy	21/04/2010
46	F	11	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	21/04/2010
47	M	10	OSA Secondary to Adenoid Hyperplasia	Tonsillectomy & Adenoidectomy	26/04/2010
48	F	25	Recurrent Tonsillitis	Tonsillectomy	28/04/2010
49	M	5	OSA Secondary to Tonsillar Hypertrophy	Tonsillectomy	03/05/2010
50	F	12	Recurrent Tonsillitis with Snoring	Tonsillectomy & Adenoidectomy	12/05/2010
51	F	6	OSA Secondary to Recurrent Tonsillitis	Tonsillectomy	31/05/2010
52	F	27	OSA secondary to Recurrent Tonsillitis	Tonsillectomy	31/05/2010
53	M	7	Chronic Tonsillitis	Tonsillectomy	02/06/2010
54	M	7	Recurrent Tonsillitis with Snoring	Tonsillectomy	09/06/2010
55	F	13	Recurrent Tonsillitis	Tonsillectomy	14/06/2010
56	M	38	Recurrent Tonsillitis with Snoring	Tonsillectomy & Adenoidectomy	14/06/2010
57	F	17	Recurrent Tonsillitis	Tonsillectomy	16/06/2010
58	F	8	Recurrent Tonsillitis	Tonsillectomy	28/06/2010
59	M	17	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	30/06/2010
60	F	7	Recurrent Tonsillitis	Tonsillectomy	30/06/2010
61	M	16	Chronic Adenotonsillitis	Tonsillectomy & Adenoidectomy	05/07/2010
62	F	36	Recurrent Tonsillitis	Tonsillectomy	05/07/2010
63	M	22	Recurrent Tonsillitis	Tonsillectomy	05/07/2010
64	F	23	Recurrent Tonsillitis	Tonsillectomy & Adenoidectomy	07/07/2010
65	F	15	OSA Secondary to Primary Snoring	Tonsillectomy & Adenoidectomy	07/07/2010
66	M	3	Chronic Tonsillitis	Tonsillectomy	14/07/2010
67	F	48	Recurrent Tonsillitis	Tonsillectomy	14/07/2010

68	M	27	Recurrent Tonsillitis	Tonsillectomy	26/07/2010
69	F	20	Recurrent Tonsillitis	Tonsillectomy	26/07/2010
70	F	30	Recurrent Tonsillitis	Tonsillectomy	02/08/2010



Tonsillar Obstructive Group
Tonsillar Infection Group
Tonsillar Infection Group

Appendix 9 Distribution and Mean of the Recovered Bacterial Isolates from Tonsillar Specimens

Patient No.	Sex	Age	Clinical Case	Tonsillar Specimen	Recovered Bacterial Isolate	Mean of Isolates per specimen
1	F	7	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus*	1
				Swab		
2	M	11	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus*	1
				Swab		
3	M	23	Recurrent Tonsillitis	Biopsy	*Enterobacter cloacae* *Acinetobacter baumannii* *Pseudomonas aeruginosa* *Staphylococcus aureus*	4
				Swab		
4	F	9	Recurrent Adenotonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	3
				Swab	*Staphylococcus aureus* *Staphylococcus aureus*	2
5	M	16	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus*	2
				Swab	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	3
6	M	31	Recurrent Tonsillitis	Biopsy	*Group B Streptococci* *Group B Streptococci* *Group G Streptococci*	3
				Swab	*Group B Streptococci* *Group B Streptococci* *Methicillin-Resistant S. aureus*	3
7	M	16	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Staphylococcus aureus* *Klebsiella pneumoniae*	3
				Swab	*Klebsiella pneumoniae* *Klebsiella pneumoniae*	2
8	M	16	Chronic Tonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Group B Streptococci* *Staphylococcus aureus* *Haemophilus parainfluenzae* *Group B Streptococci*	6
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Group F Streptococci* *Haemophilus parainfluenzae*	4
9	M	11	Recurrent Acute Tonsillitis with OSA	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Group G Streptococci* *Haemophilus parainfluenzae*	5
				Swab	*Staphylococcus aureus* *Group G Streptococci* *Haemophilus influenzae* *Group G Streptococci* *Haemophilus parainfluenzae*	5
10	F	11	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus* *Haemophilus influenzae*	6
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus*	
11	M	5	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae*	6

					Group B Streptococci *Staphylococcus aureus* *Haemophilus influenzae* *Group B Streptococci*	
				Swab	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
12	F	9	Recurrent Tonsillitis	Biopsy Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Haemophilus influenzae* *Staphylococcus aureus*	4
				Swab	*Staphylococcus aureus* *Staphylococcus aureus* *Haemophilus influenzae* *Haemophilus influenzae*	4
13	F	13	Recurrent Tonsillitis with Bilateral MEE	Biopsy	*Staphylococcus aureus* *Group G Streptococci* *Group G Streptococci* *Haemophilus influenzae* *Staphylococcus aureus*	5
				Swab	*Group G Streptococci* *Group G Streptococci*	2
14	M	9	Recurrent Tonsillitis with Bilateral MEE	Biopsy	*Staphylococcus aureus* *Group G Streptococci* *Haemophilus influenzae* *Staphylococcus aureus* *Group G Streptococci* *Haemophilus influenzae*	6
				Swab	*Group G Streptococci* *Haemophilus influenzae* *Staphylococcus aureus* *Group G Streptococci* *Haemophilus influenzae* *Staphylococcus aureus*	6
15	F	26	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Staphylococcus aureus* *Group B Streptococci* *Klebsiella pneumoniae* *Staphylococcus aureus* *Group B Streptococci*	6
				Swab	*Klebsiella pneumoniae* *Group B Streptococci* *Klebsiella pneumoniae* *Staphylococcus aureus* *Group B Streptococci*	5
16	F	24	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Staphylococcus aureus* *Group B Streptococci* *Klebsiella pneumoniae* *Staphylococcus aureus* *Group B Streptococci*	6
				Swab	*Klebsiella pneumoniae* *Staphylococcus aureus* *Klebsiella pneumoniae*	3
17	F	26	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus* *Haemophilus parainfluenzae*	3
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
18	F	17	Recurrent Tonsillitis with Bilateral MEE	Biopsy	*Haemophilus influenzae* *Haemophilus influenzae* *Group G Streptococci*	3
				Swab	*Haemophilus influenzae* *Haemophilus influenzae* *Haemophilus influenzae* *Group G Streptococci*	4
19	F	15	Recurrent Adenotonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4

				Swab	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
20	F	10	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Group G Streptococci* *Staphylococcus aureus*	3
				Swab	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
21	M	8	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Group A Streptococci*	2
				Swab	*Haemophilus influenzae* *Group A Streptococci* *Haemophilus influenzae* *Group A Streptococci*	4
22	F	5	Recurrent Tonsillitis with Snoring	Biopsy	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus*	3
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Haemophilus parainfluenzae*	3
23	F	5	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	3
				Swab	*Haemophilus influenzae* *Staphylococcus aureus*	2
24	M	6	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
				Swab	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	3
25	F	17	Chronic Tonsillitis	Biopsy	*Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci* *Haemophilus influenzae* *Staphylococcus aureus*	5
				Swab	*Group B Streptococci* *Haemophilus influenzae* *Klebsiella pneumonia*	3
26	M	7	Obstructive Sleep Apnea	Biopsy	*Group A Streptococci* *Staphylococcus aureus* *Klebsiella pneumoniae* *Group A Streptococci* *Staphylococcus aureus* *Klebsiella pneumoniae*	6
				Swab	*Group A Streptococci* *Staphylococcus aureus* *Klebsiella pneumoniae* *Group A Streptococci* *Staphylococcus aureus*	5
27	F	28	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus*	1
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus*	3
28	M	6	Adenotonsillar Hypertrophy	Biopsy	*Staphylococcus aureus* *Group B Streptococci* *Group B Streptococci* *Haemophilus parainfluenzae* *Staphylococcus aureus*	5
				Swab	*Staphylococcus aureus* *Haemophilus parainfluenzae* *Group B Streptococci* *Group B Streptococci* *Haemophilus influenzae* *Staphylococcus aureus*	6
29	F	18	Chronic Tonsillitis with Snoring	Biopsy	*Group G Streptococci* *Staphylococcus aureus* *Group G Streptococci*	5

					Staphylococcus aureus *Klebsiella pneumonia*	
				Swab	*Group G Streptococci* *Staphylococcus aureus* *Group G Streptococci* *Staphylococcus aureus*	4
30	F	24	Chronic Tonsillitis	Biopsy	*Staphylococcus aureus* *Group A Streptococci* *Staphylococcus aureus*	3
				Swab	*Group A Streptococci* *Staphylococcus aureus* *Staphylococcus aureus*	3
31	F	13	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Haemophilus influenzae*	2
				Swab	*Haemophilus influenzae* *Haemophilus influenzae*	2
32	F	12	Recurrent Tonsillitis with Bilateral MEE	Biopsy	*Haemophilus influenzae* *Staphylococcus aureus* *Staphylococcus aureus*	3
				Swab	*Haemophilus influenzae* *Staphylococcus aureus* *Staphylococcus aureus* *Streptococcus pneumoniae*	4
33	F	9	Tonsillar Hypertrophy	Biopsy	*Pseudomonas aeruginosa* *Pseudomonas aeruginosa*	2
				Swab	*Pseudomonas aeruginosa* *Haemophilus influenzae* *Pseudomonas aeruginosa*	3
34	M	8	Tonsillar Hypertrophy with OSA	Biopsy	*Haemophilus parainfluenzae* *Staphylococcus aureus* *Group F Streptococci* *Staphylococcus aureus* *Group F Streptococci*	5
				Swab	*Haemophilus parainfluenzae* *Staphylococcus aureus* *Staphylococcus aureus* *Group F Streptococci*	4
35	F	23	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Haemophilus influenzae* *Staphylococcus aureus*	3
				Swab	*Haemophilus influenzae* *Haemophilus influenzae* *Staphylococcus aureus*	3
36	F	24	Recurrent Tonsillitis	Biopsy	*Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus* *Klebsiella pneumoniae*	5
				Swab	*Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus* *Klebsiella pneumoniae*	5
37	M	11	Chronic Tonsillitis	Biopsy	*Haemophilus influenzae*	1
				Swab	*Haemophilus influenzae* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
38	M	9	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus*	1
				Swab	*Haemophilus parainfluenzae* *Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus* *Staphylococcus aureus*	5
39	F	10	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Klebsiella pneumoniae*	2
				Swab	*Klebsiella pneumoniae* *Klebsiella pneumoniae*	
40	F	27	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Group F Streptococci* *Staphylococcus aureus*	4

					Group B Streptococci	
				Swab	*Staphylococcus aureus* *Group F Streptococci* *Staphylococcus aureus* *Group B Streptococci*	4
41	F	16	Obstructive Sleep Apnea	Biopsy	*Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci*	4
				Swab	*Staphylococcus aureus* *Staphylococcus aureus*	2
42	F	8	Obstructive Sleep Apnea	Biopsy	*Haemophilus influenzae* *Staphylococcus aureus* *Group B Streptococci* *Haemophilus influenzae* *Staphylococcus aureus* *Group B Streptococci*	6
				Swab	*Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	4
43	M	10	Recurrent Tonsillitis	Biopsy	*Group B Streptococci* *Staphylococcus aureus*	2
				Swab	*Group G Streptococci* *Haemophilus parainfluenzae* *Group G Streptococci* *Haemophilus parainfluenzae* *Group G Streptococci*	5
44	M	8	Chronic Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Group C Streptococci* *Haemophilus influenzae* *Klebsiella pneumoniae* *Staphylococcus aureus* *Group C Streptococci* *Haemophilus influenzae*	7
				Swab	*Klebsiella pneumoniae* *Haemophilus influenzae* *Group C Streptococci* *Klebsiella pneumoniae* *Group C Streptococci* *Staphylococcus aureus* *Group C Streptococci* *Group B Streptococci* *Haemophilus influenzae*	9
45	M	4	Tonsillar Hypertrophy	Biopsy	*Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	4
				Swab	*Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	4
46	F	11	Tonsillar Hypertrophy	Biopsy	*Haemophilus influenzae* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci*	5
				Swab	*Haemophilus influenzae* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci*	5
47	M	10	Adenoid Hyperplasia with OSA	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae*	4
				Swab	*Staphylococcus aureus* *Klebsiella pneumoniae* *Staphylococcus aureus* *Haemophilus influenzae*	4
48	F	25	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Pseudomonas aeruginosa*	4

					Staphylococcus aureus *Pseudomonas aeruginosa*	
				Swab	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Pseudomonas aeruginosa* *Haemophilus parainfluenzae*	5
49	M	5	Tonsillar Hypertrophy with OSA	Biopsy	*Haemophilus parainfluenzae*	1
				Swab	*Haemophilus influenzae* *Haemophilus parainfluenzae*	2
50	F	12	Recurrent Tonsillitis with Snoring	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Group G Streptococci* *Staphylococcus aureus* *Haemophilus influenzae*	5
				Swab	*Staphylococcus aureus* *Staphylococcus aureus*	2
51	F	6	Recurrent Tonsillitis with OSA	Biopsy	*Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	3
				Swab	*Staphylococcus aureus* *Staphylococcus aureus* *Haemophilus influenzae*	3
52	F	27	Recurrent Tonsillitis with OSA	Biopsy	*Group B Streptococci* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus*	4
				Swab	*Group B Streptococci* *Group F Streptococci* *Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus*	5
53	M	7	Chronic Tonsillitis	Biopsy	*Citrobacter sp.* *Citrobacter sp.*	2
				Swab	*Citrobacter sp.* *Citrobacter sp.*	2
54	M	7	Recurrent Tonsillitis with Snoring	Biopsy	*Haemophilus influenzae* *Haemophilus influenzae*	2
				Swab	*Haemophilus influenzae* *Streptococcus pneumoniae* *Haemophilus influenzae*	3
55	F	13	Recurrent Tonsillitis	Biopsy	*Group A Streptococci* *Group A Streptococci*	2
				Swab Swab	*Haemophilus influenzae* *Group A Streptococci* *Group A Streptococci*	3
56	M	38	Recurrent Tonsillitis with Snoring	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus*	2
				Swab	*Staphylococcus aureus* *Staphylococcus aureus*	2
57	F	17	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae* *Group B Streptococci*	2
				Swab	*Staphylococcus aureus* *Klebsiella pneumoniae* *Staphylococcus aureus*	3
58	F	8	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus*	2
				Swab	*Staphylococcus aureus* *Group A Streptococci* *Staphylococcus aureus*	3
59	M	17	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus influenzae* *Staphylococcus aureus*	4
				Swab	*Haemophilus influenzae* *Haemophilus influenzae* *Haemophilus parainfluenzae* *Staphylococcus aureus*	4
60	F	7	Recurrent Tonsillitis	Biopsy	*Group C Streptococci* *Staphylococcus aureus* *Group C Streptococci*	4

					Staphylococcus aureus	
				Swab	*Group C Streptococci* *Staphylococcus aureus* *Group C Streptococci* *Staphylococcus aureus*	4
61	M	16	Nasopharyngeal Lymphoid Hypertrophy	Biopsy		
				Swab	*Staphylococcus aureus* *Staphylococcus aureus* *Group B Streptococci* *Group F Streptococci*	4
62	F	36	Recurrent Tonsillitis	Biopsy	*Group G Streptococci* *Staphylococcus aureus* *Group G Streptococci* *Staphylococcus aureus*	4
				Swab	*Group G Streptococci* *Staphylococcus aureus* *Group G Streptococci* *Staphylococcus aureus*	4
63	M	22	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae*	1
				Swab	*Haemophilus parainfluenzae*	1
64	F	23	Recurrent Tonsillitis	Biopsy	*Haemophilus influenzae* *Haemophilus influenzae* *Group F Streptococci* *Group B Streptococci* *Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus* *Group F Streptococci* *Group B Streptococci*	9
				Swab	*Haemophilus influenzae* *Haemophilus influenzae* *Group F Streptococci* *Group B Streptococci* *Staphylococcus aureus* *Haemophilus parainfluenzae* *Staphylococcus aureus* *Group B Streptococci*	8
65	F	15	Primary Snoring	Biopsy	*Staphylococcus aureus* *Group C Streptococci* *Group B Streptococci* *Staphylococcus aureus* *Group C Streptococci* *Group B Streptococci* *Streptococcus pneumoniae*	7
				Swab	*Staphylococcus aureus* *Group C Streptococci* *Haemophilus influenzae* *Staphylococcus aureus* *Haemophilus parainfluenzae* *Group C Streptococci*	6
66	M	3	Chronic Tonsillitis	Biopsy	*Staphylococcus aureus* *Group B Streptococci* *Haemophilus parainfluenzae* *Staphylococcus aureus*	4
				Swab	*Staphylococcus aureus* *Group B Streptococci* *Staphylococcus aureus*	3
67	F	48	Recurrent Tonsillitis	Biopsy	*Klebsiella pneumoniae*	1
				Swab		
68	M	27	Recurrent Tonsillitis	Biopsy	*Staphylococcus aureus* *Staphylococcus aureus*	2
				Swab	*Staphylococcus aureus* *Staphylococcus aureus*	2
69	F	20	Recurrent Tonsillitis	Biopsy		
				Swab		

70	**F**	**30**	**Recurrent Tonsillitis**	**Biopsy**	***Staphylococcus aureus*** ***Group B Streptococci*** ***Klebsiella pneumonia*** ***Staphylococcus aureus*** ***Klebsiella pneumonia*** ***Group B Streptococci***	**6**
				Swab	***Staphylococcus aureus*** ***Group B Streptococci*** ***Klebsiella pneumoniae*** ***Staphylococcus aureus*** ***Group B Streptococci***	**5**

The Mean Average of bacterial isolates is 3.67 isolate / tonsillar biopsy and 3.72 isolate / tonsillar swab

Tonsillar Biopsy
Tonsillar Swab
Cases with biofilms

Appendix 10 Evidence of Bacterial Biofilms Detected via CLSM among Clinical Cases

Patient No.	Sex	Age	Clinical Case	Biofilm Presence
1	F	7	Recurrent Tonsillitis	-
2	M	11	Recurrent Tonsillitis	+
3	M	23	Recurrent Tonsillitis	-
4	F	9	Recurrent Adenotonsillitis	-
5	M	16	Recurrent Tonsillitis	+
6	M	31	Recurrent Tonsillitis	+
7	M	16	Recurrent Tonsillitis	+
8	M	16	Chronic Tonsillitis	-
9	M	11	Recurrent Acute Tonsillitis with OSA	+
10	F	11	Recurrent Tonsillitis	-
11	M	5	Recurrent Tonsillitis	+
12	F	9	Recurrent Tonsillitis	+
13	F	13	Recurrent Tonsillitis with Bilateral MEE	+
14	M	9	Recurrent Tonsillitis with Bilateral MEE	-
15	F	26	Recurrent Tonsillitis	-
16	F	24	Recurrent Tonsillitis	+
17	F	26	Recurrent Tonsillitis	-
18	F	17	Recurrent Tonsillitis with Bilateral MEE	+
19	F	15	Recurrent Adenotonsillitis	+
20	F	10	Recurrent Tonsillitis	+
21	M	8	Recurrent Tonsillitis	-
22	F	5	Recurrent Tonsillitis with Snoring	+
23	F	5	Recurrent Tonsillitis	-
24	M	6	Recurrent Tonsillitis	+
25	F	17	Chronic Tonsillitis	+
26	M	7	Obstructive Sleep Apnea	-
27	F	28	Recurrent Tonsillitis	+
28	M	6	Adenotonsillar Hypertrophy	-
29	F	18	Chronic Tonsillitis with Snoring	+
30	F	24	Chronic Tonsillitis	+
31	F	13	Recurrent Tonsillitis	+
32	F	12	Recurrent Tonsillitis with Bilateral MEE	+
33	F	9	Tonsillar Hypertrophy	+
34	M	8	Tonsillar Hypertrophy with OSA	+
35	F	23	Recurrent Tonsillitis	-
36	F	24	Recurrent Tonsillitis	+
37	M	11	Chronic Tonsillitis	-
38	M	9	Recurrent Tonsillitis	-
39	F	10	Recurrent Tonsillitis	+
40	F	27	Recurrent Tonsillitis	-
41	F	16	Obstructive Sleep Apnea	+
42	F	8	Obstructive Sleep Apnea	-
43	M	10	Recurrent Tonsillitis	+
44	M	8	Chronic Tonsillitis	-
45	M	4	Tonsillar Hypertrophy	+
46	F	11	Tonsillar Hypertrophy	-
47	M	10	Adenoid Hyperplasia with OSA	+
48	F	25	Recurrent Tonsillitis	+
49	M	5	Tonsillar Hypertrophy with OSA	-
50	F	12	Recurrent Tonsillitis with Snoring	-
51	F	6	Recurrent Tonsillitis with OSA	+
52	F	27	Recurrent Tonsillitis with OSA	+
53	M	7	Chronic Tonsillitis	+
54	M	7	Recurrent Tonsillitis with Snoring	+
55	F	13	Recurrent Tonsillitis	-

56	M	38	Recurrent Tonsillitis with Snoring	-
57	F	17	Recurrent Tonsillitis	-
58	F	8	Recurrent Tonsillitis	+
59	M	17	Recurrent Tonsillitis	+
60	F	7	Recurrent Tonsillitis	-
61	M	16	Nasopharyngeal Lymphoid Hypertrophy	+
62	F	36	Recurrent Tonsillitis	-
63	M	22	Recurrent Tonsillitis	+
64	F	23	Recurrent Tonsillitis	+
65	F	15	Primary Snoring	-
66	M	3	Chronic Tonsillitis	+
67	F	48	Recurrent Tonsillitis	+
68	M	27	Recurrent Tonsillitis	+
69	F	20	Recurrent Tonsillitis	-
70	F	30	Recurrent Tonsillitis	+

Presence of biofilm in the tonsil

Biofilm was present in 42 (60%) patients whereas it was absent in 28 (40%) patients

Appendix 11 Distribution of Bacterial Isolates among Clinical Cases and their Biofilm Formation Abilities

11A *Staphylococcus aureus* (SA)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
1	Recurrent Tonsillitis	F	7	SA001		+		+	-
2	Recurrent Tonsillitis	M	11	SA002		+		+	-
3	Recurrent Tonsillitis	M	23	SA003	+		+		+
4	Recurrent Adenotonsillitis	F	9	SA004		+		+	-
				SA005				+	-
				SA006		+	+		-
				SA007		+		+	-
5	Recurrent Tonsillitis	M	16	SA008	+		+		+
				SA009		+		+	+
				SA010	+		+		+
				SA011		+		+	-
7	Recurrent Tonsillitis	M	16	SA012	+		+		+
8	Chronic Tonsillitis	M	16	SA013		+		+	-
				SA014		+		+	-
				SA015		+		+	-
9	Recurrent Acute Tonsillitis with OSA	M	11	SA016	+		+		+
				SA017	+		+		+
				SA018		+	+		+
10	Recurrent Tonsillitis	F	11	SA019		+		+	-
				SA020		+		+	-
				SA021		+		+	-
				SA022		+		+	-

11	**Recurrent Tonsillitis**	**M**	**5**	**SA023**		**+**		**+**	**-**
				SA024		**+**		**+**	**-**
				SA025		**+**	**+**		**-**
				SA026	**+**		**+**		**-**
12	**Recurrent Tonsillitis**	**F**	**9**	**SA027**		**+**		**+**	**-**
				SA028		**+**		**+**	**-**
				SA029		**+**		**+**	**-**
				SA030		**+**		**+**	**-**
13	**Recurrent Tonsillitis with Bilateral MEE**	**F**	**13**	**SA031**	**+**		**+**		**+**
				SA032	**+**		**+**		**+**
14	**Recurrent Tonsillitis with Bilateral MEE**	**M**	**9**	**SA033**	**+**		**+**		**+**
				SA034		**+**	**+**		**-**
				SA035	**+**		**+**		**-**
				SA036	**+**		**+**		**-**
15	**Recurrent Tonsillitis**	**F**	**26**	**SA037**		**+**		**+**	**-**
				SA038		**+**		**+**	**-**
				SA039		**+**		**+**	**-**
16	**Recurrent Tonsillitis**	**F**	**24**	**SA040**	**+**		**+**		**+**
				SA041	**+**		**+**		**+**
				SA042	**+**		**+**		**+**
17	**Recurrent Tonsillitis**	**F**	**26**	**SA043**		**+**		**+**	**-**
				SA044		**+**		**+**	**-**
				SA045		**+**		**+**	**-**
				SA046		**+**		**+**	**-**
19	**Recurrent Adenotonsillitis**	**F**	**15**	**SA047**	**+**		**+**		**+**
				SA048	**+**		**+**		**+**
				SA049	**+**		**+**		**+**
				SA050	**+**		**+**		**+**
21	**Recurrent Tonsillitis**	**M**	**8**	**SA051**		**+**		**+**	**-**
				SA052		**+**		**+**	**-**
				SA053		**+**		**+**	**-**
22	**Recurrent Tonsillitis**	**F**	**5**	**SA054**		**+**		**+**	**-**
				SA055		**+**		**+**	**-**

				SA056		+		+	-
24	Recurrent Tonsillitis	M	6	SA057	+		+		+
				SA058	+		+		+
				SA059	+		+		+
25	Chronic Tonsillitis	F	17	SA060		+		+	+
				SA061		+		+	+
				SA062	+		+		+
				SA063	+		+		+
26	Obstructive Sleep Apnea	M	7	SA064		+		+	-
				SA065		+		+	-
27	Recurrent Tonsillitis	F	28	SA066	+		+		+
				SA067	+		+		+
				SA068	+		+		+
				SA069		+	+		-
28	Adenotonsillar Hypertrophy	M	6	SA070		+		+	-
				SA071		+		+	-
				SA072		+		+	-
29	Chronic Tonsillitis with Snoring	F	18	SA073	+		+		+
				SA074	+		+		+
				SA075	+		+		+
				SA076		+	+		+
30	Chronic Tonsillitis	F	24	SA077	+		+		+
				SA078	+			+	-
				SA079	+		+		+
				SA080		+		+	-
31	Recurrent Tonsillitis	F	13	SA081	+		+		+
				SA082		+		+	-
				SA083	+		+		+
				SA084		+		+	-
33	Tonsillar Hypertrophy	F	9	SA085	+		+		+
				SA086	+		+		+
				SA087		+	+		-
				SA088	+		+		+
35	Recurrent Tonsillitis	F	23	SA089		+		+	-
				SA090		+		+	-

				SA091	+		+		+
				SA092		+		+	-
36	Recurrent Tonsillitis	F	24	SA093		+	+		+
				SA094	+			+	+
37	Chronic Tonsillitis	M	11	SA095		+		+	+
				SA096		+		+	+
				SA097		+	+		-
				SA098		+		+	-
38	Recurrent Tonsillitis	M	9	SA099	+			+	-
39	Recurrent Tonsillitis	F	10	SA100	+		+		+
				SA101		+		+	-
				SA102	+		+		+
				SA103		+		+	-
41	Obstructive Sleep Apnea	F	16	SA104	+		+		+
				SA105	+		+		+
				SA106	+		+		+
				SA107	+		+		+
42	Obstructive Sleep Apnea	F	8	SA112		+		+	-
				SA113	+		+		+
				SA114		+		+	-
				SA115	+		+		+
43	Recurrent Tonsillitis	M	10	SA108		+		+	+
				SA109		+		+	+
				SA110		+		+	+
				SA111		+		+	+
44	Chronic Tonsillitis	M	8	SA116		+		+	-
				SA117		+		+	-
45	Tonsillar Hypertrophy	M	4	SA118	+		+		+
				SA119	+		+		+
				SA120	+		+		+
				SA121		+	+		+
46	Tonsillar Hypertrophy	F	11	SA122		+		+	-
				SA123		+		+	-
				SA124		+		+	-
				SA125	+			+	-

47	Adenoid Hyperplasia with OSA	M	10	SA126	+		+		+
				SA127	+		+		+
				SA128	+		+		+
				SA129	+		+		+
48	Recurrent Tonsillitis	F	25	SA130		+		+	-
				SA131		+		+	-
				SA132		+		+	-
				SA133		+		+	-
50	Recurrent Tonsillitis with Snoring	F	12	SA134		+		+	-
				SA135		+		+	-
				SA136	+			+	-
				SA137		+		+	-
51	Recurrent Tonsillitis with OSA	F	6	SA138	+		+		+
				SA139	+		+		+
				SA140	+		+		+
				SA141	+		+		+
55	Recurrent Tonsillitis	F	13	SA142		+		+	-
				SA143		+		+	-
				SA144		+		+	-
				SA145		+		+	-
56	Recurrent Tonsillitis with Snoring	M	38	SA146	+			+	-
				SA147	+			+	-
57	Recurrent Tonsillitis	F	17	SA148		+		+	-
				SA149		+		+	-
				SA150		+	+		-
				SA151		+		+	-
58	Recurrent Tonsillitis	F	8	SA152	+		+		+
				SA153	+		+		+
				SA154	+		+		+
59	Recurrent Tonsillitis	M	17	SA159		+		+	-
				SA160		+		+	-
60	Recurrent Tonsillitis	F	7	SA155		+		+	-
				SA156		+		+	-
				SA157		+		-	-
				SA158		+		+	-

61	Nasopharyngeal Lymphoid Hypertrophy	M	16	SA165	+		+		+
				SA166	+		+		+
				SA167	+		+		+
				SA168	+		+		+
62	Recurrent Tonsillitis	F	36	SA161		+		+	-
				SA162		+		+	-
				SA163		+		+	-
				SA164	+		+		-
64	Recurrent Tonsillitis	F	23	SA173		+		+	-
				SA174		+		+	-
				SA175		+	+		-
				SA176	+			+	-
65	Primary Snoring	F	15	SA169		+		+	-
				SA170		+		+	-
				SA171		+		+	-
				SA172	+		+		+
68	Recurrent Tonsillitis	M	27	SA177		+		+	-
				SA178		+		+	-
				SA179		+		+	-
				SA180		+		+	-
70	Recurrent Tonsillitis	F	30	SA181	+		+		+
				SA182		+	+		-
				SA183	+		+		-
				SA184	+		+		+

Biofilm presence in the tonsils detected via CLSM
Biofilm formation *in vitro* detected via LM
Antibiotic resistant isolate

(+) indicates biofilm inhibition, (−) indicates biofilm formation
Number of *S. aureus* that are BIOFILM FORMERS = 78 (42.39%), Number of *S. aureus* that are NON-BIOFILM FORMERS = 106 (57.60%)

11B ***Haemophilus influenzae*** **(HI)**

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
4	Recurrent Adenotonsillitis	F	9	HI001		+		+	-
5	Recurrent Tonsillitis	M	16	HI002	+		+		+
7	Chronic Tonsillitis	M	16	HI003		+	+		+
9	Recurrent Acute Tonsillitis with OSA	M	11	HI004	+		+		+
				HI005	+		+		+
10	Recurrent Tonsillitis	F	11	HI006		+		+	-
11	Recurrent Tonsillitis	M	5	HI007	+		+		+
				HI008	+		+		+
				HI009		+	+		+
				HI010	+		+		+
12	Recurrent Tonsillitis	F	9	HI011	+		+		+
				HI012	+		+		+
				HI013	+		+		+
13	Recurrent Tonsillitis with Bilateral MEE	F	13	HI014	+		+		+
14	Recurrent Tonsillitis with Bilateral MEE	M	9	HI015		+		+	-
				HI016		+		+	-
				HI017		+		+	-
				HI018		+		+	-
15	Recurrent Tonsillitis	F	26	HI019	+		+		+
18	Recurrent Tonsillitis with Bilateral MEE	F	17	HI020	+		+		+
				HI021	+		+		+
				HI022		+		+	+
				HI023	+		+		+
19	Recurrent Adenotonsillitis	F	15	HI024	+			+	+
				HI025		+	+		+
				HI026	+		+		+
				HI027		+		+	+
21	Recurrent Tonsillitis	M	8	HI028	+		+		+

				HI029		+		+	-
				HI030	+		+		
22	Recurrent Tonsillitis with Snoring	F	5	HI031		+	+		+
				HI032		+	+	+	-
				HI033		+		+	-
24	Recurrent Tonsillitis	M	6	HI034	+		+		+
				HI035		+		+	-
25	Chronic Tonsillitis	F	17	HI036	+		+		+
				HI037	+		+		+
				HI038	+		+		+
26	Obstructive Sleep Apnea	M	7	HI039		+		+	-
				HI040		+		+	-
29	Chronic Tonsillitis with Snoring	F	18	HI041		+	+		+
32	Recurrent Tonsillitis with Bilateral MEE	F	12	HI042	+		+		+
				HI043		+	+		+
				HI044		+	+		+
				HI045	+		+		+
33	Tonsillar Hypertrophy	F	9	HI046		+		+	-
				HI047		+		+	-
34	Tonsillar Hypertrophy with OSA	M	8	HI048	+		+		+
36	Recurrent Tonsillitis	F	24	HI049		+	+		+
				HI050	+		+		+
				HI051	+		+		+
				HI052		+		+	-
38	Recurrent Tonsillitis	M	9	HI053		+	+		-
				HI054		+		+	-
				HI055	+		+		-
				HI056		+		+	-
42	Obstructive Sleep Apnea	F	8	HI057	+			+	-
				HI058		+		+	-
				HI059		+		+	-
				HI060		+		+	-
45	Tonsillar Hypertrophy	M	4	HI061	+		+		+
				HI062	+		+		+
				HI063	+		+		+

46	**Tonsillar Hypertrophy**	**F**	**11**	**HI064**	**+**		**+**		**+**
				HI065		**+**		**+**	**-**
47	**Adenoid Hyperplasia with OSA**	**M**	**10**	**HI066**		**+**		**+**	**-**
				HI067	**+**		**+**		**+**
				HI068	**+**		**+**		**+**
49	**Tonsillar Hypertrophy with OSA**	**M**	**5**	**HI069**		**+**		**+**	**-**
50	**Recurrent Tonsillitis with Snoring**	**F**	**12**	**HI070**		**+**		**+**	**-**
				HI071	**+**		**+**		**+**
53	**Chronic Tonsillitis**	**M**	**7**	**HI072**		**+**		**+**	**+**
				HI073		**+**	**+**		**+**
				HI074		**+**		**+**	**+**
				HI075	**+**		**+**		**+**
54	**Recurrent Tonsillitis with Snoring**	**M**	**7**	**HI076**		**+**		**+**	**-**
58	**Recurrent Tonsillitis**	**F**	**8**	**HI077**	**+**		**+**		**+**
				HI078	**+**		**+**		**+**
				HI079		**+**		**+**	**+**
				HI080	**+**		**+**		**+**
61	**Nasopharyngeal Lymphoid Hypertrophy**	**M**	**16**	**HI081**	**+**		**+**		**+**
				HI082	**+**		**+**		**+**
				HI083	**+**		**+**		**+**
				HI084	**+**		**+**		**+**
63	**Recurrent Tonsillitis**	**M**	**22**	**HI085**	**+**		**+**		**+**
65	**Recurrent Tonsillitis**	**F**	**15**	**HI086**		**+**		**+**	**-**

Number of *H. influenzae* that are BIOFILM FORMERS = 55 (63.95%), Number of *H. influenzae* that are NON-BIOFILM FORMERS = 31 (36.04%)

11C *Streptococcus agalactiae* (GBS)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
6	Recurrent Tonsillitis	M	31	STB01		+		+	-
				STB02		+		+	-
				STB03		+		+	-
				STB04		+		+	-
7	Chronic Tonsillitis	M	16	STB05	+		+		+
				STB06	+		+		+
11	Recurrent Tonsillitis	M	5	STB07	+		+		+
				STB08	+			+	+
15	Recurrent Tonsillitis	F	26	STB09		+		+	-
				STB10		+		+	-
				STB11		+	+		-
				STB12		+		+	-
16	Recurrent Tonsillitis	F	24	STB13		+	+		-
				STB14		+		+	-
				STB15		+		+	-
26	Obstructive Sleep Apnea	M	7	STB16		+		+	-
				STB17		+		+	-
				STB18		+		+	-
29	Chronic Tonsillitis with Snoring	F	18	STB19		+		+	-
				STB20	+		+		+
				STB21		+	+		+
				STB22	+		+		+
37	Chronic Tonsillitis	M	11	STB23		+		+	-
				STB24		+		+	-
				STB25		+		+	-
				STB26	+		+		-
41	Obstructive Sleep Apnea	F	16	STB27		+		+	-

				STB28	+		+		-
42	Obstructive Sleep Apnea	F	8	STB31		+		+	-
				STB32		+		+	-
43	Recurrent Tonsillitis	M	10	STB29		+	+		+
				STB30	+		+		+
44	Chronic Tonsillitis	M	8	STB33		+		+	-
46	Tonsillar Hypertrophy	F	11	STB34		+		+	-
				STB35	+		+		-
				STB36		+		+	-
				STB37	+		+		-
51	Recurrent Tonsillitis with OSA	F	6	STB38	+		+		+
				STB39	+		+		+
				STB40	+		+		+
				STB41	+		+		+
56	Recurrent Tonsillitis with Snoring	M	38	STB42		+		+	-
59	Recurrent Tonsillitis	M	17	STB43		+		+	-
61	Nasopharyngeal Lymphoid Hypertrophy	M	16	STB44	+		+		+
				STB45	+		+		+
				STB46		+	+		+
				STB47	+		+		+
64	Recurrent Tonsillitis	F	23	STB50		+		+	-
				STB51		+		+	-
65	Primary Snoring	F	15	STB48		+		+	-
				STB49	+			+	-
68	Recurrent Tonsillitis	M	27	STB52		+		+	-
70	Recurrent Tonsillitis	F	30	STB53	+		+		+
				STB54		+		+	-
				STB55	+		+		+
				STB56		+	+		+

Number of *Streptococcus agalactiae* that are BIOFILM FORMERS = 19 (33.92%), Number of *Streptococcus agalactiae* that are NON-BIOFILM FORMERS = 37 (66.07%)

11D *Haemophilus parainfluenzae* (HP)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
8	Chronic Tonsillitis	M	16	HP001	+		+		-
				HP002		+		+	-
				HP003		+		+	-
9	Recurrent Acute Tonsillitis with OSA	M	11	HP004	+		+		+
				HP005		+	+		+
10	Recurrent Tonsillitis	F	11	HP006		+		+	-
12	Recurrent Tonsillitis	F	9	HP007	+		+		+
15	Recurrent Tonsillitis	F	26	HP008		+		+	-
				HP009		+		+	-
23	Recurrent Tonsillitis	F	5	HP010		+	+		-
				HP011		+		+	-
				HP012	+			+	-
28	Adenotonsillar Hypertrophy	M	6	HP013		+		+	-
29	Chronic Tonsillitis with Snoring	F	18	HP014	+		+		+
				HP015	+		+		+
35	Recurrent Tonsillitis	F	23	HP016		+		+	-
				HP017	+			+	-
39	Recurrent Tonsillitis	F	10	HP018	+		+		+
				HP019	+		+		+
44	Chronic Tonsillitis	M	8	HP020		+	+		+
				HP021	+		+		+
45	Tonsillar Hypertrophy	M	4	HP022	+		+		+
48	Recurrent Tonsillitis	F	25	HP023		+	+		+
49	Tonsillar Hypertrophy with OSA	M	5	HP024		+		+	-
				HP025		+		+	-
58	Recurrent Tonsillitis	F	8	HP026	+		+		+

61	**Nasopharyngeal Lymphoid Hypertrophy**	**M**	**16**	**HP027**	**+**		**+**		**+**
				HP028		**+**	**+**		**+**
63	**Recurrent Tonsillitis**	**M**	**22**	**HP029**	**+**		**+**		**+**
64	**Recurrent Tonsillitis**	**F**	**23**	**HP030**		**+**		**+**	**+**
65	**Primary Snoring**	**F**	**15**	**HP031**		**+**	**+**		**-**

Number of *H. parainfluenzae* that are BIOFILM FORMERS = 16 (51.61%), Number of *H. parainfluenzae* that are NON-BIOFILM FORMERS = 15 (48.38%)

11E *Klebsiella pneumoniae* (KP)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
5	**Recurrent Tonsillitis**	**M**	**16**	**KP001**	**+**		**+**		**+**
				KP002		**+**		**+**	**-**
				KP003	**+**		**+**		**+**
				KP004	**+**		**+**		**+**
15	**Recurrent Tonsillitis**	**F**	**26**	**KP005**		**+**		**+**	**-**
				KP006		**+**		**+**	**-**
				KP007	**+**			**+**	**-**
				KP008		**+**		**+**	**-**
16	**Recurrent Tonsillitis**	**F**	**24**	**KP009**	**+**		**+**		**+**

				KP010		+	+		+
				KP011	+		+		+
				KP012	+		+		+
26	Obstructive Sleep Apnea	M	7	KP013		+		+	-
27	Recurrent Tonsillitis	F	28	KP014	+		+		+
				KP015	+		+		+
				KP016		+	+		+
30	Chronic Tonsillitis	F	24	KP017	+		+		+
37	Chronic Tonsillitis	M	11	KP018		+		+	-
				KP019	+		+		-
47	Adenoid Hyperplasia with OSA	M	10	KP020		+		+	-
52	Recurrent Tonsillitis with OSA	F	27	KP021	+		+		+
				KP022		+	+		+
				KP023	+		+		+
				KP024	+		+		+
56	Recurrent Tonsillitis with Snoring	M	38	KP025		+		+	-
				KP026		+		+	-
67	Recurrent Tonsillitis	F	48	KP027		+		+	-
70	Recurrent Tonsillitis	F	30	KP028		+	+		-
				KP029		+		+	-
				KP030	+			+	-

Number of *K. pneumoniae* that are BIOFILM FORMERS = 15(50%), Number of *K. pneumoniae* that are NON-BIOFILM FORMERS = 15(50%)

11F Group F Streptococci (GFS)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
8	Chronic Tonsillitis	M	16	STF01		+		+	-
35	Recurrent Tonsillitis	F	23	STF02	+			+	-
				STF03		+		+	-
				STF04		+		+	-
41	Obstructive Sleep Apnea	F	16	STF05		+		+	-
				STF06		+	+		-
51	Recurrent Tonsillitis with OSA	F	6	STF07	+		+		+
59	Recurrent Tonsillitis	M	17	STF08	+		+		-
61	Nasopharyngeal Lymphoid Hypertrophy	M	16	STF09		+		+	-
				STF10		+		+	-
				STF11	+		+		+

Number of Group F Streptococci (GFS) that are BIOFILM FORMERS = 2 (18.18%), Number of Group F Streptococci (GFS) that are NON-BIOFILM FORMERS = 9 (81.81%)

11G Group G Streptococci (GGS)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
6	Recurrent Tonsillitis	M	31	STG01		+		+	-
9	Recurrent Acute Tonsillitis with OSA	M	11	STG02	+		+		-
				STG03		+		+	-
				STG04		+		+	-
13	Recurrent Tonsillitis with Bilateral MEE	F	13	STG05	+		+		+
				STG06		+	+		+
				STG07		+		+	-
				STG08	+		+		+
14	Recurrent Tonsillitis with Bilateral MEE	M	9	STG09		+		+	-
				STG10		+		+	-
				STG11	+		+		-
				STG12		+		+	-
18	Recurrent Tonsillitis with Bilateral MEE	F	17	STG13	+			+	-
30	Chronic Tonsillitis	F	24	STG14	+		+		+
				STG15		+	+		+
				STG16	+			+	-
				STG17		+	+		+
44	Chronic Tonsillitis	M	8	STG18		+		+	-
				STG19		+	+		-
				STG20		+		+	-
50	Recurrent Tonsillitis with Snoring	F	12	STG21	+			+	-
62	Recurrent Tonsillitis	F	36	STG22		+		+	-
				STG23	+		+		-
				STG24		+		+	-
				STG25		+		+	-

Number of Group G Streptococci (GGS) that are BIOFILM FORMERS = 6(24%), Number of Group G Streptococci (GGS) that are NON-BIOFILM FORMERS = 19 (76%)

11H *Streptococcus pyogenes* (GABHS)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
22	Recurrent Tonsillitis with Snoring	F	5	STA01	+		+		+
				STA02		+		+	-
				STA03	+		+		+
31	Recurrent Tonsillitis	F	13	STA04	+		+		+
				STA05		+	+		+
54	Recurrent Tonsillitis with Snoring	M	7	STA06	+		+		+
				STA07	+		+		+
				STA08		+		+	-
				STA09	+		+		+
57	Recurrent Tonsillitis	F	17	STA10		+		+	+
58	Recurrent Tonsillitis	F	8	STA11	+		+		+
				STA12	+		+		+
				STA13		+	+		+
				STA14	+		+		+

GABHS = Group A Beta-Haemolytic Streptococci

Number of *S. pyogenes* (GABHS) that are BIOFILM FORMERS = 12(85.71%), Number of *S. pyogenes* (GABHS) that are NON-BIOFILM FORMERS = 2 (14.28%)

11I ***Pseudomonas aeruginosa*** **(PA)**

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
3	Recurrent Tonsillitis	M	23	PA001		+		+	-
34	Tonsillar Hypertrophy with OSA	M	8	PA002	+		+		+
				PA003		+		+	-
				PA004	+		+		+
				PA005	+		+		+
48	Recurrent Tonsillitis	F	25	PA006	+		+		+
				PA007	+		+		+
				PA008	+		+		+
				PA009	+		+		+

Number of *P. aeruginosa* that are BIOFILM FORMERS = 7 (77.77%), Number of *P. aeruginosa* that are NON-BIOFILM FORMERS = 2 (22.22%)

11J Group C Streptococci (GCS)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
1	Recurrent Tonsillitis	F	7	STC01		+		+	-
				STC02	+		+		+
				STC03		+		+	-
				STC04	+		+		+
65	Primary Snoring	F	15	STC05		+		+	-
				STC06		+		+	-
				STC07		+	+		-
				STC08		+		+	-

Number of Group C Streptococci (GCS) that are BIOFILM FORMERS = 2 (25%), Number of Group C Streptococci (GCS) that are NON-BIOFILM FORMERS = 6 (75%)

11K Methicillin Resistant *S. aureus* (MRSA)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
6	Recurrent Tonsillitis	M	31	MRS01	+		+		+

Number of MRSA that are BIOFILM FORMERS = 1 (100%), Number of MRSA that are NON-BIOFILM FORMERS = 0

11L *Citrobacter* sp.

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
52	Recurrent Tonsillitis with OSA	F	27	CBF01		+		+	-
				CBF02	+			+	-
				CBF03		+		+	-
				CBF04		+		+	-

Number of *Citrobacter* sp. that are BIOFILM FORMERS = 0, Number of *Citrobacter* sp. that are NON-BIOFILM FORMERS = 4 (100%)

11M *Streptococcus pneumoniae* (SPn)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
33	Tonsillar Hypertrophy	F	9	STP01		+		+	+
53	Chronic Tonsillitis	M	7	STP02	+		+		+
65	Primary Snoring	F	15	STP03		+		+	-

Number of *S. pneumoniae* (SPn) that are BIOFILM FORMERS = 2 (66.66%), Number of *S. pneumoniae* (SPn) that are NON-BIOFILM FORMERS = 1 (33.33%)

11N *Enterobacter cloacae* (EC)

Patient No.	Clinical Case	Sex	Age	Clinical Isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
3	Recurrent Tonsillitis	M	23	EBC01		+		+	-

Number of *Enterobacter cloacae* that are BIOFILM FORMERS = 0, Number of *Enterobacter cloacae* that are NON-BIOFILM FORMERS = 1 (100%)

11O *Acinetobacter baumannii* (AB)

Patient No.	Clinical Case	Sex	Age	Clinical isolate No.	Biofilm Formation				
					Congo Red Agar method (CRA)		Microtitter Plate assay (MTP)		Microscopic Observation of Biofilm Formation
					High	Weak	High	Weak	
3	Recurrent Tonsillitis	M	23	ABB01	+			+	-

Number of *Acinetobacter baumannii (AB)* that are BIOFILM FORMERS = 0, Number of *Acinetobacter baumannii (AB)* that are NON-BIOFILM FORMERS = 1(100%)

Appendix 12 Antimicrobial Susceptibility Results and Antibiogram Patterns of Tonsillar Isolates

12A *Staphylococcus aureus* (SA)

Antimicrobial Class	Penicillins[1]	Aminoglycosides[2]	Macrolides[2]	Folate pathway inhibitors[2]	Lincosamides[2]	Fusidane[2]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Methicillin (ME)	Gentamycin (GM)	Erythromycin (EM)	Co-trimoxazole (SXT) [a]	Clindamycin (CM)	Fusidic Acid (FA)[3]	
Disk Content	5 µg	10 µg	15 µg	1.25/23.75 µg	2 µg	10 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 14 – R ≤ 9	S ≥ 15 – R ≤ 12	S ≥ 23 – R ≤ 13	S ≥ 16 – R ≤ 10	S ≥ 21 – R ≤ 14	S ≥22 – R ≤19	
Clinical Isolate							
SA001	S	S	S	S	S	S	SSSSSS
SA002	S	S	S	S	S	S	SSSSSS
SA003	S	S	S	S	S	S	SSSSSS
SA004	S	S	S	S	S	S	SSSSSS
SA005	S	S	S	S	S	S	SSSSSS
SA006	S	S	S	S	S	S	SSSSSS
SA007	S	S	S	S	S	S	SSSSSS
SA008	S	S	S	S	S	R	SSSSSR
SA009	S	S	S	S	S	R	SSSSSR
SA010	S	S	S	S	S	R	SSSSSR
SA011	S	S	S	S	S	R	SSSSSR
SA012	S	S	S	S	S	S	SSSSSS
SA013	S	S	S	S	S	S	SSSSSS
SA014	S	S	S	S	S	S	SSSSSS
SA015	S	S	S	S	S	S	SSSSSS
SA016	S	S	S	S	S	R	SSSSSR
SA017	S	S	S	S	S	R	SSSSSR
SA018	S	S	S	S	S	R	SSSSSR
SA019	S	S	S	S	S	S	SSSSSS
SA020	S	S	S	S	S	S	SSSSSS
SA021	S	S	S	S	S	S	SSSSSS
SA022	S	S	S	S	S	S	SSSSSS
SA023	S	S	S	S	S	S	SSSSSS

SA024	S	S	S	S	S	S	SSSSSS
SA025	S	S	S	S	S	S	SSSSSS
SA026	S	S	S	S	S	S	SSSSSS
SA027	S	S	S	S	S	S	SSSSSS
SA028	S	S	S	S	S	S	SSSSSS
SA029	S	S	S	S	S	S	SSSSSS
SA030	S	S	S	S	S	S	SSSSSS
SA031	S	S	S	S	S	S	SSSSSS
SA032	S	S	S	S	S	S	SSSSSS
SA033	S	S	S	S	S	S	SSSSSS
SA034	S	S	S	S	S	S	SSSSSS
SA035	S	S	S	S	S	S	SSSSSS
SA036	S	S	S	S	S	S	SSSSSS
SA037	S	S	S	S	S	S	SSSSSS
SA038	S	S	S	S	S	S	SSSSSS
SA039	S	S	S	S	S	S	SSSSSS
SA040	S	S	S	S	S	S	SSSSSS
SA041	S	S	S	S	S	S	SSSSSS
SA042	S	S	S	S	S	S	SSSSSS
SA043	S	S	S	S	S	S	SSSSSS
SA044	S	S	S	S	S	S	SSSSSS
SA045	S	S	S	S	S	S	SSSSSS
SA046	S	S	S	S	S	S	SSSSSS
SA047	S	S	S	S	S	S	SSSSSS
SA048	S	S	S	S	S	S	SSSSSS
SA049	S	S	S	S	S	S	SSSSSS
SA050	S	S	S	S	S	S	SSSSSS
SA051	S	S	S	S	S	S	SSSSSS
SA052	S	S	S	S	S	S	SSSSSS
SA053	S	S	S	S	S	S	SSSSSS
SA054	S	S	S	S	S	S	SSSSSS
SA055	S	S	S	S	S	S	SSSSSS
SA056	S	S	S	S	S	S	SSSSSS
SA057	S	S	S	S	S	S	SSSSSS
SA058	S	S	S	S	S	S	SSSSSS
SA059	S	S	S	S	S	S	SSSSSS
SA060	S	S	S	S	S	S	SSSSSS
SA061	S	S	S	S	S	S	SSSSSS
SA062	S	S	S	S	S	S	SSSSSS
SA063	S	S	S	S	S	S	SSSSSS
SA064	S	S	S	S	S	R	SSSSSR

SA065	S	S	S	S	S	R	SSSSSR
SA066	S	S	S	S	S	S	SSSSSS
SA067	S	S	S	S	S	S	SSSSSS
SA068	S	S	S	S	S	S	SSSSSS
SA069	S	S	S	S	S	S	SSSSSS
SA070	S	S	S	S	S	S	SSSSSS
SA071	S	S	S	S	S	S	SSSSSS
SA072	S	S	S	S	S	S	SSSSSS
SA073	S	S	S	S	S	S	SSSSSS
SA074	S	S	S	S	S	S	SSSSSS
SA075	S	S	S	S	S	S	SSSSSS
SA076	S	S	S	S	S	S	SSSSSS
SA077	S	S	S	S	S	S	SSSSSS
SA078	S	S	S	S	S	S	SSSSSS
SA079	S	S	S	S	S	S	SSSSSS
SA080	S	S	S	S	S	S	SSSSSS
SA081	S	S	S	S	S	S	SSSSSS
SA082	S	S	S	S	S	S	SSSSSS
SA083	S	S	S	S	S	S	SSSSSS
SA084	S	S	S	S	S	S	SSSSSS
SA085	S	S	S	S	S	S	SSSSSS
SA086	S	S	S	S	S	S	SSSSSS
SA087	S	S	S	S	S	S	SSSSSS
SA088	S	S	S	S	S	S	SSSSSS
SA089	S	S	S	S	S	S	SSSSSS
SA090	S	S	S	S	S	S	SSSSSS
SA091	S	S	S	S	S	S	SSSSSS
SA092	S	S	S	S	S	S	SSSSSS
SA093	S	S	S	S	S	S	SSSSSS
SA094	S	S	S	S	S	S	SSSSSS
SA095	S	S	S	S	S	S	SSSSSS
SA096	S	S	S	S	S	S	SSSSSS
SA097	S	S	S	S	S	S	SSSSSS
SA098	S	S	S	S	S	S	SSSSSS
SA099	S	S	S	S	S	S	SSSSSS
SA100	S	S	S	S	S	S	SSSSSS
SA101	S	S	S	S	S	S	SSSSSS
SA102	S	S	S	S	S	S	SSSSSS
SA103	S	S	S	S	S	S	SSSSSS
SA104	S	S	S	S	S	S	SSSSSS
SA105	S	S	S	S	S	S	SSSSSS

SA106	S	S	S	S	S	S	SSSSSS
SA107	S	S	S	S	S	S	SSSSSS
SA108	S	S	S	S	S	S	SSSSSS
SA109	S	S	S	S	S	S	SSSSSS
SA110	S	S	S	S	S	S	SSSSSS
SA111	S	S	S	S	S	S	SSSSSS
SA112	S	S	S	S	S	S	SSSSSS
SA113	S	S	S	S	S	R	SSSSSR
SA114	S	S	S	S	S	R	SSSSSR
SA115	S	S	S	S	S	R	SSSSSR
SA116	S	S	S	S	S	S	SSSSSS
SA117	S	S	S	S	S	S	SSSSSS
SA118	S	S	S	S	S	S	SSSSSS
SA119	S	S	S	S	S	S	SSSSSS
SA120	S	S	S	S	S	S	SSSSSS
SA121	S	S	S	S	S	S	SSSSSS
SA122	S	S	S	S	S	S	SSSSSS
SA123	S	S	S	S	S	S	SSSSSS
SA124	S	S	S	S	S	S	SSSSSS
SA125	S	S	S	S	S	S	SSSSSS
SA126	S	S	S	S	S	S	SSSSSS
SA127	S	S	S	S	S	S	SSSSSS
SA128	S	S	S	S	S	S	SSSSSS
SA129	S	S	S	S	S	S	SSSSSS
SA130	S	S	S	S	S	S	SSSSSS
SA131	S	S	S	S	S	S	SSSSSS
SA132	S	S	S	S	S	S	SSSSSS
SA133	S	S	S	S	S	S	SSSSSS
SA134	S	S	S	S	S	S	SSSSSS
SA135	S	S	S	S	S	S	SSSSSS
SA136	S	S	S	S	S	S	SSSSSS
SA137	S	S	S	S	S	S	SSSSSS
SA138	S	S	S	S	S	R	SSSSSR
SA139	S	S	S	S	S	R	SSSSSR
SA140	S	S	S	S	S	R	SSSSSR
SA141	S	S	S	S	S	R	SSSSSR
SA142	S	S	S	S	S	S	SSSSSS
SA143	S	S	S	S	S	S	SSSSSS
SA144	S	S	S	S	S	S	SSSSSS
SA145	S	S	S	S	S	S	SSSSSS
SA146	S	S	S	S	S	S	SSSSSS

SA147	S	S	S	S	S	S	SSSSSS
SA148	S	S	S	S	S	S	SSSSSS
SA149	S	S	S	S	S	S	SSSSSS
SA150	S	S	S	S	S	S	SSSSSS
SA151	S	S	S	S	S	S	SSSSSS
SA152	S	S	S	S	S	S	SSSSSS
SA153	S	S	S	S	S	S	SSSSSS
SA154	S	S	S	S	S	S	SSSSSS
SA155	S	S	S	S	S	S	SSSSSS
SA156	S	S	S	S	S	S	SSSSSS
SA157	S	S	S	S	S	S	SSSSSS
SA158	S	S	S	S	S	S	SSSSSS
SA159	S	S	S	S	S	S	SSSSSS
SA160	S	S	S	S	S	S	SSSSSS
SA161	S	S	S	S	S	R	SSSSSR
SA162	S	S	S	S	S	R	SSSSSR
SA163	S	S	S	S	S	R	SSSSSR
SA164	S	S	S	S	S	R	SSSSSR
SA165	S	S	S	S	S	S	SSSSSS
SA166	S	S	S	S	S	S	SSSSSS
SA167	S	S	S	S	S	S	SSSSSS
SA168	S	S	S	S	S	S	SSSSSS
SA169	S	S	S	S	S	S	SSSSSS
SA170	S	S	S	S	S	S	SSSSSS
SA171	S	S	S	S	S	S	SSSSSS
SA172	S	S	S	S	S	S	SSSSSS
SA173	S	S	S	S	S	S	SSSSSS
SA174	S	S	S	S	S	S	SSSSSS
SA175	S	S	S	S	S	S	SSSSSS
SA176	S	S	S	S	S	S	SSSSSS
SA177	S	S	S	S	S	S	SSSSSS
SA178	S	S	S	S	S	S	SSSSSS
SA179	S	S	S	S	S	S	SSSSSS
SA180	S	S	S	S	S	S	SSSSSS
SA181	S	S	S	S	S	S	SSSSSS
SA182	S	S	S	S	S	S	SSSSSS
SA183	S	S	S	S	S	S	SSSSSS
SA184	S	S	S	S	S	S	SSSSSS

[a] Co-trimoxazole = Trimethoprim-sulfamethoxazole
[1]β-Lactam antibiotic, [2]Non–β-Lactam antibiotic,
[3]Results are based on the zone diameter proposed interpretive breakpoints for fusidic acid against *Staphylococcus aureus* (Jones et al 2010).

Data were generated from clinical isolates of patients' specimens submitted to the microbiology referral laboratory at University Malaya Medical Centre (UMMC).

Results are based on the zone diameter interpretive standards for ***Staphylococcus aureus*** from the CLSI Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement M100-S20 Vol. 30 No. 1, **Page 60-68**, 2010.

Antibiotic resistant isolate

Susceptibility Testing Conditions:

Test method: Disk diffusion.

Medium: Mueller-Hinton Agar (MHA).

Inoculum: Direct colony suspension, equivalent to a 0.5 McFarland standard.

12B Methicillin Resistant *S. aureus* (MRSA)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Folate pathway inhibitors[2]	Fusidane[2]	Aminoglycosides[2]	Lincosamides[2]	Ansamycins	Quinolones[2]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Methicillin (ME)	Erythromycin (EM)	Co-trimoxazole (SXT) [a]	Fusidic Acid (FA)[3]	Gentamycin (GM)	Clindamycin (CM)	Rifampin (RA)	Ciprofloxacin (CIP)	
Disk Content	5µg	15µg	1.25/23.75µg	10µg	10µg	2µg	5µg	5µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 14 – R ≤ 9	S ≥ 23 – R ≤ 13	S ≥ 16 – R ≤ 10	S ≥22 – R ≤19	S ≥ 15 – R ≤ 12	S ≥ 21 – R ≤ 14	S ≥ 20 – R ≤ 16	S ≥ 21 – R ≤ 15	
Clinical Isolate									
SA001	R	R	S	S	R	S	S	R	RRSSRSSR

12C *Streptococcus pyogenes* (GABHS)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Glycopeptides[2]	Penicillins[1]	Penems[1]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Lincosamides[2]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Penicillin (PEN)	Erythromycin (EM)	Vancomycin (VA)	Ampicillin (AM)	Imipenem (IPM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX)	Clindamycin (CM)	Cefuroxime (CXM)[4]	
Disk Content	10 µg	15 µg	30 µg	10 µg	10 µg	1.25/23.75 µg	30 µg	2 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 24 – R ≤	S ≥ 21 – R ≤ 15	S ≥ 17 – R ≤ –	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ 24 – R ≤ –	
Clinical Isolate										
STA01	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STA02	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA03	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA04	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA05	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA06	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA07	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA08	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA09	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STA10	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA11	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STA12	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA13	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STA14	S	S	S	S	S	R	S	S	S	SSSSSRSSS

GABHS = Group A Beta-Haemolytic Streptococci

12D *Streptococcus agalactiae* (GBS)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Glycopeptides[2]	Penicillins[1]	Penems[1]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Lincosamides[2]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Penicillin (PEN)	Erythromycin (EM)	Vancomycin (VA)	Ampicillin (AM)	Imipenem (IPM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX) [b]	Clindamycin (CM)	Cefuroxime (CXM)[4]	
Disk Content	10 µg	15 µg	30 µg	10 µg	10 µg	1.25/23.75 µg	30 µg	2 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 24 – R ≤	S ≥ 21 – R ≤ 15	S ≥ 17 – R ≤ –	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ 24 – R ≤ –	
Clinical Isolate										
STB01	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB02	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB03	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB04	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB05	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB06	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB07	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB08	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB09	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB10	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB11	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB12	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB13	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB14	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB15	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB16	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB17	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB18	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB19	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB20	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB21	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB22	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB23	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB24	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB25	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB26	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB27	S	S	S	S	S	R	S	S	S	SSSSSRSSS

STB28	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB29	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB30	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB31	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB32	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB33	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STB34	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB35	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB36	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB37	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB38	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB39	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB40	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB41	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB42	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB43	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB44	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB45	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB46	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB47	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB48	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB49	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB50	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB51	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB52	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB53	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB54	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB55	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STB56	S	S	S	S	S	R	S	S	S	SSSSSRSSS

For the ***Streptococcus spp. β-Hemolytic Group***, an organism that is susceptible to penicillin can be considered susceptible to the listed antimicrobial agents when used for approved indications and need not be tested against those agents. For β-hemolytic streptococci (Groups A, B, C, G): ampicillin, amoxicillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, cefazolin, cefepime, cephradine, **cephalothin**, cefotaxime, ceftriaxone, ceftizoxime, **imipenem**, ertapenem, and meropenem. In addition, for group A streptococci only: cefaclor, cefdinir, cefprozil, ceftibuten, cefuroxime, **cefpodoxime**, and cephapirin (CLSI document M100-S20 Vol. 30 No. 1, **Page 92-94**, 2010). Results are based on the zone diameter interpretive standards for ***Streptococcus spp. β-Hemolytic Group*** from the CLSI Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement M100-S20 Vol. 30 No. 1, **Page 92-94**, 2010.

12E Group C Streptococci (GCS)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Glycopeptides[2]	Penicillins[1]	Penems[1]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Lincosamides[2]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Penicillin (PEN)	Erythromycin (EM)	Vancomycin (VA)	Ampicillin (AM)	Imipenem (IPM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX)	Clindamycin (CM)	Cefuroxime (CXM)[4]	
Disk Content	10 µg	15 µg	30 µg	10 µg	10 µg	1.25/23.75 µg	30 µg	2 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 24 – R ≤	S ≥ 21 – R ≤ 15	S ≥ 17 – R ≤ –	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ 24 – R ≤	
Clinical Isolate										
STC01	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC02	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC03	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC04	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC05	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC06	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC07	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STC08	S	S	S	S	S	S	S	S	S	SSSSSSSSS

12F Group F Streptococci (GFS)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Glycopeptides[2]	Penicillins[1]	Penems[1]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Lincosamides[2]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Penicillin (PEN)	Erythromycin (EM)	Vancomycin (VA)	Ampicillin (AM)	Imipenem (IPM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX)	Clindamycin (CM)	Cefuroxime (CXM)[4]	
Disk Content	10 µg	15 µg	30 µg	10 µg	10 µg	1.25/23.75 µg	30 µg	2 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 24 – R ≤	S ≥ 21 – R ≤ 15	S ≥ 17 – R ≤ –	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ 24 – R ≤ –	
Clinical Isolate										
STF01	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF02	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF03	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF04	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF05	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF06	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF07	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF08	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF09	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF10	S	S	S	S	S	S	S	S	S	SSSSSSSS
STF11	S	S	S	S	S	S	S	S	S	SSSSSSSS

Viridans Group Streptococci (VGS) include the following five groups, with several species within each group: mutans group, salivarius group, bovis group, anginosus group (previously "S. milleri" group), and mitis group. The anginosus group includes small colony–forming β-hemolytic strains with groups A, C, F, and G antigens. For detailed information on the species within the groups, please refer to recent clinical microbiology literature (CLSI 2010).

12G Group G Streptococci (GGS)

Antimicrobial Class	Penicillins[1]	Macrolides[2]	Glycopeptides[2]	Penicillins[1]	Penems[1]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Lincosamides[2]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Penicillin (PEN)	Erythromycin (EM)	Vancomycin (VA)	Ampicillin (AM)	Imipenem (IPM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX)	Clindamycin (CM)	Cefuroxime (CXM)[4]	
Disk Content	10 µg	15 µg	30 µg	10 µg	10 µg	1.25/23.75 µg	30 µg	2 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 24 – R ≤	S ≥ 21 – R ≤ 15	S ≥ 17 – R ≤ –	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 19 – R ≤ 15	S ≥ 24 – R ≤ –	
Clinical Isolate										
STG01	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG02	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG03	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG04	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG05	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG06	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG07	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG08	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG09	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG10	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG11	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG12	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG13	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG14	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG15	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG16	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG17	S	S	S	S	S	R	S	S	S	SSSSSRSSS
STG18	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG19	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG20	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG21	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG22	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG23	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG24	S	S	S	S	S	S	S	S	S	SSSSSSSSS
STG25	S	S	S	S	S	S	S	S	S	SSSSSSSSS

12H *Streptococcus pneumoniae* (SPn)

Antimicrobial Class	Macrolides[2]	Folate pathway inhibitors[2]	Cephems (oral)[1]	Cephems (parenteral)[1]	Penicillins[1]	Glycopeptides[2]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Erythromycin (EM)	Co-trimoxazole (SXT) [a]	Cephalexin (LEX)[3]	Cefuroxime (CXM)[4]	Penicillin (PEN)	Vancomycin (VA)	
Disk Content	15 µg	1.25/23.75 µg	30 µg	30 µg	10 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 21 – R ≤ 15	S ≥ 19 – R ≤ 15	S ≥ – – R ≤	S ≥ 24 – R ≤	S ≥ – – R ≤	S ≥ 17 – R ≤	
Clinical Isolate							
STP01	R	R	R	S	R	S	RRRSRS
STP02	R	R	R	S	R	S	RRRSRS
STP03	R	R	R	S	R	S	RRRSRS

12I *Haemophilus influenzae* (HI)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	Cephems (oral)[1]	Cephems (parenteral)[1]	Macrolides[2]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Amoxicillin-Clavulanic acid (AMC)	Cefuroxime (CXM)	Ceftriaxone (CTR)[b]	Azithromycin (AZM)	
Disk Content	10 µg	1.25/23.75µg	20/10 µg	30 µg	30 µg	15 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 22 – R ≤ 18	S ≥ 16 – R ≤ 10	S ≥ 20 – R ≤ 19	S ≥ 20 – R ≤ 16	S ≥ 26 – R –	S ≥ 12 – R –	
Clinical Isolate							
HI001	S	S	S	S	S	S	SSSSSS
HI002	S	S	S	S	S	S	SSSSSS
HI003	S	S	S	S	S	S	SSSSSS
HI004	S	S	S	S	S	S	SSSSSS
HI005	S	R	S	S	S	S	SRSSSS
HI006	S	S	S	S	S	S	SSSSSS
HI007	R	S	S	S	S	S	RSSSSS
HI008	R	S	S	S	S	S	RSSSSS
HI009	S	S	S	S	S	S	SSSSSS
HI010	S	S	S	S	S	S	SSSSSS
HI011	S	S	S	S	S	S	SSSSSS
HI012	S	S	S	S	S	S	SSSSSS
HI013	S	R	S	S	S	S	SRSSSS
HI014	S	R	S	S	S	S	SRSSSS
HI015	S	S	S	S	S	S	SSSSSS
HI016	S	S	S	S	S	S	SSSSSS
HI017	S	S	S	S	S	S	SSSSSS
HI018	S	S	S	S	S	S	SSSSSS
HI019	S	R	S	S	S	S	SRSSSS
HI020	S	R	S	S	S	S	SRSSSS
HI021	S	R	S	S	S	S	SRSSSS
HI022	S	R	S	S	S	S	SRSSSS
HI023	S	R	S	S	S	S	SRSSSS
HI024	S	S	S	S	S	S	SSSSSS
HI025	S	S	S	S	S	S	SSSSSS
HI026	S	S	S	S	S	S	SSSSSS
HI027	S	S	S	S	S	S	SSSSSS

HI028	S	R	S	S	S	S	SRSSSS
HI029	S	R	S	S	S	S	SRSSSS
HI030	S	R	S	S	S	S	SRSSSS
HI031	S	S	S	S	S	S	SSSSSS
HI032	S	S	S	S	S	S	SSSSSS
HI033	S	S	S	S	S	S	SSSSSS
HI034	S	R	S	S	S	S	SRSSSS
HI035	S	S	S	S	S	S	SSSSSS
HI036	S	S	S	S	S	S	SSSSSS
HI037	S	S	S	S	S	S	SSSSSS
HI038	R	S	S	S	S	S	RSSSSS
HI039	S	S	S	S	S	S	SSSSSS
HI040	S	S	S	S	S	S	SSSSSS
HI041	S	R	S	S	S	S	SRSSSS
HI042	S	S	S	S	S	S	SSSSSS
HI043	S	S	S	S	S	S	SSSSSS
HI044	S	S	S	S	S	S	SSSSSS
HI045	S	S	S	S	S	S	SSSSSS
HI046	S	S	S	S	S	S	SSSSSS
HI047	S	S	S	S	S	S	SSSSSS
HI048	R	R	R	S	S	S	RRRSSS
HI049	R	R	S	S	S	S	RRSSSS
HI050	R	R	S	S	S	S	RRSSSS
HI051	S	S	S	S	S	S	SSSSSS
HI052	S	S	S	S	S	S	SSSSSS
HI053	S	S	S	S	S	S	SSSSSS
HI054	S	S	S	S	S	S	SSSSSS
HI055	S	S	S	S	S	S	SSSSSS
HI056	S	S	S	S	S	S	SSSSSS
HI057	S	S	S	S	S	S	SSSSSS
HI058	S	S	S	S	S	S	SSSSSS
HI059	S	S	S	S	S	S	SSSSSS
HI060	S	S	S	S	S	S	SSSSSS
HI061	S	R	S	S	S	S	SRSSSS
HI062	S	R	S	S	S	S	SRSSSS
HI063	S	R	S	S	S	S	SRSSSS
HI064	R	S	S	S	S	S	RSSSSS
HI065	S	S	S	S	S	S	SSSSSS
HI066	S	S	S	S	S	S	SSSSSS
HI067	S	R	S	S	S	S	SRSSSS
HI068	S	R	S	S	S	S	SRSSSS

HI069	S	S	S	S	S	S	SSSSSS
HI070	S	R	S	S	S	S	SRSSSS
HI071	S	R	S	S	S	S	SRSSSS
HI072	S	S	S	S	S	S	SSSSSS
HI073	S	S	S	S	S	S	SSSSSS
HI074	S	S	S	S	S	S	SSSSSS
HI075	S	S	S	S	S	S	SSSSSS
HI076	S	S	S	S	S	S	SSSSSS
HI077	S	R	S	S	S	S	SRSSSS
HI078	S	R	S	S	S	S	SRSSSS
HI079	S	R	S	S	S	S	SRSSSS
HI080	S	R	S	S	S	S	SRSSSS
HI081	R	S	S	S	S	S	RSSSSS
HI082	R	S	S	S	S	S	RSSSSS
HI083	R	S	S	S	S	S	RSSSSS
HI084	R	S	S	S	S	S	RSSSSS
HI085	R	S	S	S	S	S	RSSSSS
HI086	S	S	S	S	S	S	SSSSSS

Amoxicillin-clavulanic acid, azithromycin, clarithromycin, cefaclor, cefprozil, loracarbef, cefdinir, cefixime, cefpodoxime, cefuroxime, and telithromycin are oral agents that may be used as empiric therapy for respiratory tract infections due to Haemophilus spp. The results of susceptibility tests with these antimicrobial agents are often not useful for management of individual patients. However, susceptibility testing of Haemophilus spp. with these compounds may be appropriate for surveillance or epidemiological studies.

12J *Haemophilus parainfluenzae* (HP)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	Cephems (oral)[1]	Cephems (parenteral)[1]	Macrolides[2]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Amoxicillin-Clavulanic acid (AMC)	Cefuroxime (CXM)	Ceftriaxone (CTR)	Azithromycin (AZM)	
Disk Content	10 µg	1.25/23.75 µg	20/10 µg	30 µg	30 µg	15 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 22 – R ≤ 18	S ≥ 16 – R ≤ 10	S ≥ 20 – R ≤ 19	S ≥ 20 – R ≤ 16	S ≥ 26 – R –	S ≥ 12 – R –	
Clinical Isolate							
HP001	S	R	S	S	S	S	SRSSSS
HP002	S	S	S	S	S	S	SSSSSS
HP003	S	S	S	S	S	S	SSSSSS
HP004	S	S	S	S	S	S	SSSSSS
HP005	S	S	S	S	S	S	SSSSSS
HP006	S	R	S	S	S	S	SRSSSS
HP007	S	S	S	S	S	S	SSSSSS
HP008	S	R	S	S	S	S	SRSSSS
HP009	S	R	S	S	S	S	SRSSSS
HP010	S	R	S	S	S	S	SRSSSS
HP011	R	R	S	S	S	S	RRSSSS
HP012	R	S	S	S	S	S	RSSSSS
HP013	S	R	S	S	S	S	SRSSSS
HP014	S	S	S	S	S	S	SSSSSS
HP015	S	R	S	S	S	S	SRSSSS
HP016	S	S	S	S	S	S	SSSSSS
HP017	S	S	S	S	S	S	SSSSSS
HP018	S	S	S	S	S	S	SSSSSS
HP019	S	S	S	S	S	S	SSSSSS
HP020	S	S	S	S	S	S	SSSSSS
HP021	S	S	S	S	S	S	SSSSSS
HP022	S	S	S	S	S	S	SSSSSS
HP023	S	S	S	S	S	S	SSSSSS
HP024	S	S	S	S	S	S	SSSSSS
HP025	S	R	S	S	S	S	SRSSSS
HP026	S	S	S	S	S	S	SSSSSS

HP027	S	S	S	S	S	S	SSSSSS
HP028	S	S	S	S	S	S	SSSSSS
HP029	S	R	S	S	S	S	SRSSSS
HP030	S	S	S	S	S	S	SSSSSS
HP031	S	S	S	S	S	S	SSSSSS

12K *Klebsiella pneumoniae* (KP)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	β-lactamase inhibitor combinations[1]	Aminoglycosides[2]	Aminoglycosides[2]	Cephems (parenteral)[1]
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Ampicillin-Sulbactam (SAM)	Amoxicillin-Clavulanic Acid (AMC)	Gentamycin (GM)	Amikacin (AMK)	Cefuroxime (CXM)
Disk Content	10 µg	1.25/23.75 µg	10/10 µg	20/10 µg	10 µg	30 µg	30 µg
Zone Diameter Breakpoints, nearest whole mm	S ≥ 17 – R ≤ 13	S ≥ 19 – R ≤ 15	S ≥ 15 – R ≤ 11	S ≥ 18 – R ≤ 13	S ≥ 15 – R ≤ 12	S ≥ 17 – R ≤ 14	S ≥ 18 – R ≤ 14
Clinical Isolate							
KP001	R	S	S	S	S	S	S
KP002	R	S	S	S	S	S	S
KP003	R	S	S	S	S	S	S
KP004	R	S	S	S	S	S	S
KP005	R	S	S	S	S	S	S
KP006	R	S	S	S	S	S	S
KP007	R	S	S	S	S	S	S
KP008	R	S	S	S	S	S	S
KP009	R	S	S	S	S	S	S
KP010	R	S	S	S	S	S	S
KP011	R	S	S	S	S	S	S
KP012	R	S	S	S	S	S	S
KP013	R	S	S	S	S	S	S
KP014	R	S	S	S	S	S	S
KP015	R	S	S	S	S	S	S
KP016	R	S	S	S	S	S	S

KP017	R	S	S	S	S	S	S
KP018	R	S	S	S	S	S	S
KP019	R	S	S	S	S	S	S
KP020	R	S	S	S	S	S	S
KP021	R	S	S	S	S	S	S
KP022	R	S	S	S	S	S	S
KP023	R	S	S	S	S	S	S
KP024	R	S	S	S	S	S	S
KP025	R	S	S	S	S	S	S
KP026	R	S	S	S	S	S	S
KP027	R	S	S	S	S	S	S
KP028	R	S	S	S	S	S	S
KP029	R	S	S	S	S	S	S
KP030	R	S	S	S	S	S	S

Antimicrobial Class	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Quinolones[2]	Penems[1]	β-lactamase inhibitor combinations[1]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Cefoperazone (CFP)	Ceftazidime (CAZ)[b]	Ceftriaxone (CTR)	Ciprofloxacin (CIP)	Imipenem (IPM)	Piperacillin-Tazobactam (TZP)	Cefotaxime (CTX)[b]	
Disk Content	75 µg	30 µg	30 µg	5µg	10 µg	100/10 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 21 – R ≤ 15	S ≥ 21 – R ≤ 17	S ≥ 23 – R ≤ 19	S ≥ 21 – R ≤ 15	S ≥ 16 – R ≤ 13	S ≥ 21 – R ≤ 17	S ≥ 26 – R ≤ 22	
Clinical Isolate								
KP001	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP002	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP003	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP004	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP005	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP006	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP007	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP008	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP009	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP010	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP011	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP012	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP013	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP014	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP015	S	S	S	S	S	S	S	RSSSSSSSSSSSS

KP016	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP017	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP018	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP019	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP020	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP021	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP022	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP023	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP024	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP025	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP026	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP027	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP028	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP029	S	S	S	S	S	S	S	RSSSSSSSSSSSS
KP030	S	S	S	S	S	S	S	RSSSSSSSSSSSS

12L *Pseudomonas aeruginosa* (PA)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	β-lactamase inhibitor combinations[1]	Aminoglycosides[2]	Aminoglycosides[2]	Cephems (parenteral)[1]
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Ampicillin-Sulbactam (SAM)	Amoxicillin-Clavulanic Acid (AMC)	Gentamicin (GM)	Amikacin (AMK)	Cefuroxime (CXM)
Disk Content	10 µg	1.25/23.75 µg	10/10 µg	20/10 µg	10 µg	30 µg	30 µg
Zone Diameter Breakpoints, nearest whole mm	S ≥ 17 – R ≤ 13	S ≥ 19 – R ≤ 15	S ≥ 15 – R ≤ 11	S ≥ 18 – R ≤ 13	S ≥ 15 – R ≤ 12	S ≥ 17 – R ≤ 14	S ≥ 18 – R ≤ 14
Clinical Isolate							
PA001	R	S	R	R	R	R	S
PA002	R	S	R	R	R	R	S
PA003	R	S	R	R	R	R	S
PA004	R	S	R	R	R	R	S
PA005	R	S	R	R	R	R	S
PA006	R	S	R	R	R	R	S
PA007	R	S	R	R	R	R	S
PA008	S	S	S	S	S	S	S
PA009	S	S	S	S	S	S	S

Antimicrobial Class	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Quinolones[2]	Penems[1]	β-lactamase inhibitor combinations[1]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Cefoperazone (CFP)	Ceftazidime (CAZ) [b]	Ceftriaxone (CTR)	Ciprofloxacin (CIP)	Imipenem (IPM)	Piperacillin-Tazobactam (TZP)	Cefotaxime (CTX) [b]	
Disk Content	75 µg	30 µg	30 µg	5µg	10 µg	100/10 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 21 – R ≤ 15	S ≥ 18 – R ≤ 14	S ≥ 21 – R ≤ 13	S ≥ 21 – R ≤ 15	S ≥ 16 – R ≤ 13	S ≥ 18 – R ≤ 17	S ≥ 23 – R ≤ 14	
Clinical Isolate								
PA001	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA002	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA003	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA004	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA005	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA006	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA007	S	S	S	S	S	S	S	RSRRRRSSSSSSSS
PA008	S	S	S	S	S	S	S	SSSSSSSSSSSSS
PA009	S	S	S	S	S	S	S	SSSSSSSSSSSSS

12M *Citrobacter* sp.

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	β-lactamase inhibitor combinations[1]	Aminoglycosides[2]	Aminoglycosides[2]	Cephems (parenteral)[1]
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Ampicillin-Sulbactam (SAM)	Amoxicillin-Clavulanic Acid (AMC)	Gentamicin (GM)	Amikacin (AMK)	Cefuroxime (CXM)
Disk Content	10 µg	1.25/23.75 µg	10/10 µg	20/10 µg	10 µg	30 µg	30 µg
Zone Diameter Breakpoints, nearest whole mm	S ≥ 17 – R ≤ 13	S ≥ 19 – R ≤ 15	S ≥ 15 – R ≤ 11	S ≥ 18 – R ≤ 13	S ≥ 15 – R ≤ 12	S ≥ 17 – R ≤ 14	S ≥ 18 – R ≤ 14
Clinical Isolate							
CBF01	R	S	S	S	S	S	S
CBF02	R	S	S	S	S	S	S
CBF03	R	S	S	S	S	S	S
CBF04	R	S	S	S	S	S	S

Antimicrobial Class	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Quinolones[2]	Penems[1]	β-lactamase inhibitor combinations[1]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Cefoperazone (CFP)	Ceftazidime (CAZ) [b]	Ceftriaxone (CTR)	Ciprofloxacin (CIP)	Imipenem (IPM)	Piperacillin-Tazobactam(TZP)	Cefotaxime (CTX) [b]	
Disk Content	75 µg	30 µg	30 µg	5µg	10 µg	100/10 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 21 – R ≤ 15	S ≥ 18 – R ≤ 14	S ≥ 21 – R ≤ 13	S ≥ 21 – R ≤ 15	S ≥ 16 – R ≤ 13	S ≥ 18 – R ≤ 17	S ≥ 23 – R ≤ 14	
Clinical Isolate								
CBF01	S	S	S	S	S	S	S	RSSSSSSSSSSSS
CBF02	S	S	S	S	S	S	S	RSSSSSSSSSSSS
CBF03	S	S	S	S	S	S	S	RSSSSSSSSSSSS
CBF04	S	S	S	S	S	S	S	RSSSSSSSSSSSS

12N *Enterobacter cloacae* (EC)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	β-lactamase inhibitor combinations[1]	Aminoglycosides[2]	Aminoglycosides[2]	Cephems (parenteral)[1]
Antimicrobial Agent Generic Name (Abbreviation)	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Ampicillin-Sulbactam (SAM)	Amoxicillin-Clavulanic Acid (AMC)	Gentamycin (GM)	Amikacin (AMK)	Cefuroxime (CXM)
Disk Content	10 µg	1.25/23.75 µg	10/10 µg	20/10 µg	10 µg	30 µg	30 µg
Zone Diameter Breakpoints, nearest whole mm	S ≥ 17 – R ≤ 13	S ≥ 19 – R ≤ 15	S ≥ 15 – R ≤ 11	S ≥ 18 – R ≤ 13	S ≥ 15 – R ≤ 12	S ≥ 17 – R ≤ 14	S ≥ 18 – R ≤ 14
Clinical Isolate							
EBC01	R	S	S	R	S	S	S

Antimicrobial Class	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Quinolones[2]	Penems[1]	β-lactamase inhibitor combinations[1]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Agent Generic Name (Abbreviation)	Cefoperazone (CFP)	Ceftazidime (CAZ) [b]	Ceftriaxone (CTR)	Ciprofloxacin (CIP)	Imipenem (IPM)	Piperacillin-Tazobactam (TZP)	Cefotaxime (CTX) [b]	
Disk Content	75 µg	30 µg	30 µg	5µg	10 µg	100/10 µg	30 µg	
Zone Diameter Breakpoints, nearest whole mm	S ≥ 21 – R ≤ 15	S ≥ 18 – R ≤ 14	S ≥ 21 – R ≤ 13	S ≥ 21 – R ≤ 15	S ≥ 16 – R ≤ 13	S ≥ 18 – R ≤ 17	S ≥ 23 – R ≤ 14	
Clinical Isolate								
EBC01	S	S	S	S	S	S	S	RSSRSSSSSSSSS

12O *Acinetobacter baumannii* (AB)

Antimicrobial Class	Penicillins[1]	Folate pathway inhibitors[2]	β-lactamase inhibitor combinations[1]	β-lactamase inhibitor combinations[1]	Aminoglycosides[2]	Aminoglycosides[2]	Cephems (parenteral)[1]
Antimicrobial Generic Name	Ampicillin (AM)	Co-trimoxazole (SXT) [a]	Ampicillin-Sulbactam (SAM)	Amoxicillin-Clavulanic Acid (AMC)	Gentamycin (GM)	Amikacin (AMK)	Cefuroxime (CXM)
Disk Content	10 µg	1.25/23.75 µg	10/10 µg	20/10 µg	10 µg	30 µg	30 µg
Zone Diameter Breakpoints	S ≥ 17 – R ≤ 13	S ≥ 19 – R ≤ 15	S ≥ 15 – R ≤ 11	S ≥ 18 – R ≤ 13	S ≥ 15 – R ≤ 12	S ≥ 17 – R ≤ 14	S ≥ 18 – R ≤ 14
Clinical Isolate							
ABB01	R	S	S	R	S	S	R

Antimicrobial Class	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Cephems (parenteral)[1]	Quinolones[2]	Penems[1]	β-lactamase inhibitor combinations[1]	Cephems (parenteral)[1]	Antibiogram pattern
Antimicrobial Generic Name	Cefoperazone (CFP)	Ceftazidime (CAZ) [b]	Ceftriaxone (CTR)	Ciprofloxacin (CIP)	Imipenem (IPM)	Piperacillin-Tazobactam (TZP)	Cefotaxime (CTX) [b]	
Disk Content	75 µg	30 µg	30 µg	5µg	10 µg	100/10 µg	30 µg	
Zone Diameter Breakpoints	S ≥ 21 – R ≤ 15	S ≥ 18 – R ≤ 14	S ≥ 21 – R ≤ 13	S ≥ 21 – R ≤ 15	S ≥ 16 – R ≤ 13	S ≥ 18 – R ≤ 17	S ≥ 23 – R ≤ 14	
Clinical Isolate								
ABB01	S	S	R	S	S	S	R	RSSRSSRSSRSSSR